Research Article

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A Platform for Characterizing Therapeutic Monoclonal Antibody Breakdown Products by 2D Chromatography and Top-Down Mass Spectrometry

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Abstract. With the growing commercialization of therapeutic monoclonal antibodies developed for the treatment of various diseases comes the need for increased analytical scrutiny of the impurity components contained within such drug products. Traditionally, relatively low performance and throughput analytical techniques were employed for elucidating the product-related breakdown components derived from the original molecule, including N-terminal Edman sequencing and matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry. Although N-terminal sequencing provides a definitive starting point of an unknown breakdown product, the resolution and mass accuracy of MALDI-TOF instruments are often insufficient for unambiguous sequence characterization. Described here is the implementation of existing advanced analytical technologies, including high-performance mass spectrometry (LTQ-Orbitrap XL-ETD) and a chip-based nanoelectrospray autosampling robot (TriVersa NanoMate), for the thorough identification and characterization of breakdown products derived from a force-degraded monoclonal antibody. Many anticipated breakdown products were identified, including Fab fragment (48,325 Da) and heavy chain polypeptide hydrolysis product (15,521 Da). Using high-resolution collisionally induced and electron transfer dissociation methods, additional identifications were made with specific localization of unpredicted modifications. As examples, a modified Fab fragment (N- and C-terminal cyclization, 47,902 Da) and a hydrolyzed free light chain impurity components (23,191 Da) were identified with a high degree of confidence (*E* value, <1e-5). This work describes the approach for top-down characterization of breakdown products and is readily applicable to additional monoclonal antibodies (mAb) characterization experiments, including charge isoform characterization and aggregate analysis, for a more thorough understanding of therapeutic mAb drug products.

KEY WORDS: 2D chromatography; liquid chromatography-mass spectrometry (LC-MS); tandem mass spectrometry (MS/MS); therapeutic monoclonal antibodies.

INTRODUCTION

The increasingly successful development of therapeutic monoclonal antibodies (mAb) efficacious for the treatment of various human diseases, ranging from cancer to autoimmune disorders, has provided many new drug treatment options to patients suffering from such conditions (1). With the commercial manufacture of new therapeutic drug products comes the need for complete characterization of the molecular variants that may affect overall product quality and safety (2). Unlike their small molecule counterparts, therapeutic

mAbs are generally more complex due to their overall large size (150 kDa), multichain structure (tetramer, IgG), and critical protein folding that is attributed to protein binding and efficacy (3). Further, mAbs frequently display a high degree of structural heterogeneity, including glycosylation, C-terminal processing, oxidation, deamidation, hydrolysis, disulfide scrambling, in addition to possible codon-specific impurities (4,5). As a result, increased analytical scrutiny is required to characterize not only the intended protein form, but also any product variants that may be present in the final drug product (5).

The Food and Drug Administration, the European Medicines Agency (6), and collectively the International Conference on Harmonization (7) guidance regarding drug product safety and efficacy recommend a thorough process- and product-related impurity characterization prior to issuance of a Biologics License Application. Process-related impurities are generally a result of incomplete purification of product during the manufacturing process and are typically characterized using well-established biochemical methods (8),

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including Western blotting (host cell protein contaminants and hydrolysis products) (9), PCR amplification and sequencing (DNA/RNA impurities) (10,11), and GC-MS/liquid chromatography-mass spectrometry (LC-MS; leachables, small molecule contaminants) (12). On the other hand, product-related impurities represent the components derived from the original drug product that result from some chemical or physical change of the therapeutic mAb (7). Typically, these are composed of protein aggregates, precursor products (*i.e.*, signal peptides), modified antibody, or truncation products. Because product-related impurities are derived from the original mAb product and are relatively low in concentrations, more advanced analytical techniques are often required for their analysis. The use of high-performance mass spectrometry has provided a powerful and direct means for elucidating specific subsets of various impurity components, including charge isoforms (13), sequence variants (14), and even protein aggregates (15). Clearly, these applications demonstrate a desire for more informative analysis of impurity components which may pose a significant risk to the safety and efficacy of the final drug product (7,16).

Product stability and breakdown component characterization experiments are typically performed using stressed studies, such as incubating products at elevated temperatures or under UV light exposure (17). Both physical (aggregation and precipitation) and chemical (*e.g.*, hydrolysis, oxidation, and deamidation) modifications of the mAb molecule are common impurity products of such stability stress experiments (18). There currently exists a variety of bioanalytical methods other than mass spectrometry better suited for the routine isolation and relative quantitation of high-molecular weight protein aggregates (covalently and noncovalently linked) (19,20). Unlike very large protein aggregates, identifying chemically modified products by mass spectrometry is ideal because these resulting products are typically relatively small and/or lower in mass. Prior to the widespread availability of high-performance mass spectrometers, matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by N-terminal Edman sequencing had been the sequencing method of choice to infer impurity identification (21). With only the intact mass information, however, these approaches were insufficient to provide unambiguous sequence assignment of hydrolysis products. Additionally, subtle modifications resulting from degradation (*e.g.*, methionine oxidation) required relatively low throughput peptide mapping experiments to monitor individual sequences affected. Despite advancements in high-performance liquid chromatography (HPLC)/ultraperformance liquid chromatography (22) and sequencing technologies (23), routine analysis requires significant time resources.

The accessibility of high-performance mass spectrometers has provided a more straightforward means for achieving high-confidence mAb breakdown product identification. Current commercial instruments provide very high mass accuracy (typically <2 ppm) and resolving power (typically in excess of 60,000), thereby reducing the number of possible candidate impurity matches. Additionally, most instruments provide for high-performance tandem mass spectrometry experiments (*i.e.*, tandem mass spectrometry; MS/MS), including electron

capture (24) or electron transfer (25) dissociation techniques, that allow gas-phase sequencing of unknown impurities. These mass spectrometry based methods, performed “top-down” on the intact (*i.e.*, nondigested) polypeptide level (26), retain the specific structural information lost by “bottom-up” approaches. Taken together, the direct assessment of chromatographically separated impurity components without the need for complementary sequencing experiments (*e.g.*, Edman sequencing) provides a level of both throughput and information not obtainable by “traditional” approaches.

There exists a variety of mass spectrometry tools that can be directly applied for developing a robust platform for characterizing mAb breakdown products. Although online LC-MS allows detection of eluting impurity species, obtaining high-resolution top-down tandem mass spectrometry (MSⁿ) information is not routinely achievable on a chromatographic time scale. Front-end sampling instruments such as the Triversa NanoMate provide additional options for capturing MS/MS data not easily obtained from online LC-MS experiments (27,28). Using split-flow chromatography, online nanoelectrospray LC-MS can be acquired with concurrent fraction collection, enabling >99.9% of eluting impurity components to be preserved. Resulting fractions can then be thoroughly interrogated offline by tandem mass spectrometry experiments using the instruments semi-automated, direct infusion sampling capabilities. Increasing the MS/MS analysis time from only a few seconds using online techniques to tens of minutes with the offline approach improves data quality and subsequent identification confidence. All acquired mass spectrometry data can then be processed by existing software tools, such as ProMass and ProsightPC v2.0 (29), which is currently being used by the proteomics community to convert raw full scan and MS/MS data into intact mass and sequence identifications, respectively.

Described here is the implementation of such a mass spectrometry-based impurity characterization platform, in which stress-induced drug samples were fractionated by 2D chromatography. Antibody breakdown components were tentatively identified by intact mass analysis from online LC-MS, and unambiguously assigned by MS/MS analysis of collected components. The analysis was semi-automated with limited operator intervention, and total data collection time was approximately 1 week. This platform was intentionally designed to readily accommodate alternative characterization analyses within the same analytical framework (*e.g.*, top-down charge isoform characterization by 2D ion exchange, reverse-phase chromatography LC-MS). The work demonstrated here is the most accurate and straightforward approach towards the characterization of therapeutic mAb breakdown products, and is well-positioned to be used for more extensive studies (*e.g.*, elucidation of breakdown mechanism).

MATERIALS AND METHODS

Materials

Recombinant IgG1 antibodies produced from NSO cells were purified in house according to established protocols.

Test mAb product was subjected to stability challenge by incubating at 37–42°C for 13 months in the dark. The samples were then stored at 2–8°C until analysis. These stability mAbs samples (10 mg/mL) were desalted using NAP-5 column (GE Healthcare, Piscataway, NJ, USA), aliquoted, and taken to dryness prior to chromatographic separation.

Size-exclusion Chromatography of Heat-stressed mAb Samples

One milligram of heat stressed mAb was resuspended in 100 μ L of deionized water and subjected to size-exclusion chromatography (SEC) separation using the following parameters: column, Tosoh-Haas, TSK Gel 3000SW_{XL}, 300 $\times$ 7.8 mm operated at ambient temperature ($\sim$ 23°C), flow rate 0.5 mL/min, buffer 20 mM sodium phosphate (pH 7.0)+0.5 M NaCl, detection at 214 and 280 nm. Eluting fractions were collected every 0.66 min. Nine replicate 1 mg injections were performed, their chromatographic profiles compared, and their respective fractions combined. Fractions (30 total) were analyzed by SDS-PAGE, and “high” (roughly >100 kDa), “mid” (roughly 100–50 kDa), and “low” (roughly <60 kDa) molecular weight fractions were combined and concentrated using Amicon Ultra-15 (Millipore, Billerica, MA, USA) concentrators (MWCO 10,000) according to the manufacturers protocol. A bicinchoninic acid protein assay was performed to determine fraction concentrations, and the final SEC fraction purity analyzed by analytical SDS-PAGE.

Online, Split-flow Reverse-phase Chromatography of SEC Fractions

Approximately 200 μ g of each SEC fraction was injected onto a C8 reverse-phase column (150 $\times$ 2.1 mm, 3 μ m, Agilent Technologies, Palo Alto, CA, USA) operating at 80°C with buffers 0.2% formic acid+0.05% TFA/water (solvent A) and 0.2% formic acid+0.05% TFA/acetonitrile (solvent B). Samples were gradient separated (25%B for 7 min, gradient of 0.34%B/min over next 23 min, then gradient 15.5%B/min for 4 min, hold at 95%B for 5 min) using Agilent HP1200 HPLC system coupled to a TriVersa Nanomate robot (Advion, Ithaca, NY, USA). The liquid chromatography flow was split prior to the electrospray ionization interface at roughly 350 nL to the mass spectrometer and 399.6 μ L to a fraction collector. Seventy reverse-phase fractions (2 min⁻¹) were collected for each of the three SEC fractions for a total of 210 fractions. Each LC-MS separation (with fraction collection) of all three SEC fractions was replicated to allow reduced and nonreduced analysis of each fraction (see below). The split flow was infused into Thermo LTQ-Orbitrap XL-ETD mass spectrometer operating with source conditions as follows: 1.6 kV capillary voltage, 105 V tube lens, 240°C capillary temperature.

Direct Infusion MS/MS of Reverse-phase Fractions

All reverse-phase fractions were taken to dryness *via* speedvac prior to semi-automated MS/MS analysis. Fractions to be analyzed under nonreducing conditions were

resuspended in 20 μ L of electrospray buffer (50:49:1; acetonitrile/water/formic acid) and aliquoted to individual wells of a 384 sample plate. Analogous fractions analyzed under reducing conditions were resuspended in reducing buffer (100 mM dithiothreitol in 100 mM ammonium bicarbonate and 6 M guanidine-HCL), incubated at 50°C for 20 min, and C₄ ZipTip (Millipore) desalted prior to analysis. Ten microliter of each sample was aspirated and directly infused into the LTQ-Orbitrap XL-ETD mass spectrometer using the TriVersa Nanomate operating at 1.55 kV source voltage and 0.6 psi. Automated MS/MS was performed using methods created specifically for “low” and “high” molecular weights. For the low molecular weight method, one full-scan spectra was acquired (600–2,000 m/z at 60,000 resolving power), followed by the collisionally induced and electron transfer dissociation of the top three most abundant ions (each 60,000 resolving power and 25 μ scans). For the high molecular weight method, two full-scan spectra were acquired (400–2,000 m/z at 60,000 resolving power and 600–4,000 m/z at 7,500 resolving power), followed by collisionally induced dissociation of the top three most abundant ions (each 60,000 resolving power and 25 μ scans), followed by one full-scan source fragmentation spectrum (75 V source, 60,000 resolving power, 25 μ scans). All samples infused for 5 min prior to the start of the mass spectrometric analysis to ensure consistent electrospray infusion and were subsequently analyzed by the mass spectrometer for 15 min. Dynamic exclusion was employed to allow for fragmentation of multiple ion components using the following parameters: repeat count, 1; repeat duration, 30 s; exclusion duration, 300 s; exclusion list, 500; exclusion mass width, $\pm$ 5 m/z.

Full-scan MS Data Deconvolution using ProMass

Collected online LC-MS spectra were processed by ProMass for Xcalibur v2.5 (Thermo Scientific, Waltham, MA, USA) to produce average masses of eluting polypeptide components. Chromatographic “peaks” (*i.e.*, time ranges) assigned by Xcalibur Processing method ($\sim$ 100 total chromatographic times, between 9 and 35 min) were individually deconvoluted by ProMass using the following parameters: *m/z* range, 700–4,000; output mass, 10–160 kDa, baseline removal on at 0.7; peak width=3; merge width=0.3; smooth width=7; number of smooths=2; noise threshold=2 S/N. Deconvoluted spectra were manually inspected for accuracy.

High-resolution MS/MS Data Analysis using ProSightPC v2.0

Collected high-resolution MS/MS spectra of selected reverse-phase fractions were analyzed by ProSightPC v2.0 (Thermo Scientific, Waltham, MA, USA) using both high-throughput mode and search tree logic. MS/MS spectra were searched against the mAb sequence using 2.2 Da and 25 ppm tolerances for intact and fragment masses, respectively. Those spectra not matching within pre-defined tolerance (*i.e.*, *E* value $\leq$ 1e-4 and *P* score $\leq$ 1e-4) were searched using 25 Da and 25 ppm tolerances for intact and fragment masses, respectively. Matching identifications were manually inspected for accuracy.

RESULTS

A semi-automated platform was developed to provide complete and confident characterization of mAb breakdown products, including not only proper sequence assignment but thorough characterization of any modifications which may have occurred during the sample degradation. Combining existing technologies in the field of protein mass spectrometry, this platform applies front-end chromatographic separation, mass

analysis, and gas-phase sequencing for the characterization of each breakdown component detectable within the single forced-degradation sample, with little operator intervention (Fig. 1). Size-exclusion chromatography was first used to prefractionate samples and enrich for lower molecular weight breakdown products. A second dimension of C8 reverse-phase chromatography was performed using split flow, with a portion directed to the mass spectrometer instrument for online, intact mass information, and a major portion directed to fraction collection.

Fig. 1. mAb breakdown product characterization platform. Force-degraded monoclonal antibody sample is fractionated by size-exclusion chromatography prior to online reverse-phase LC-MS of resultant SEC fractions. Using the TriVersa Nanomate split-flow capabilities, a portion of the eluting components are directed to the LTQ-Orbitrap XL-ETD for online detection while the majority is collected into microplate wells. The collected fractions are then analyzed under both reduced and nonreduced conditions by direct infusion nanoelectrospray and data-dependant high-resolution tandem mass spectrometry. Intact mass and MS/MS sequencing information is analyzed by the software tools of Promass and ProsightPC, respectively

Subsequent analysis of collected fractions was performed in a semi-automated fashion using a chip-based autosampler infusion robot (Triversa Nanomate), and sequencing information was obtained through tandem mass spectrometry experiments. The raw data was converted to intact mass and characterization information with the software tools of ProMass for Xcalibur (*i.e.*, mass deconvolution) and ProsightPC (*i.e.*, MS/MS sequence characterization).

Detailed characterization of impurity components requires the analytical ability to span the dynamic range inherent to both the precursor and hydrolysis products present. As only ~15% of the force-degraded sample results in many different hydrolysis products (see Fig. 2b, unfractionated), front-end chromatographic separations are needed to enrich for such products. Semi-preparative SEC fractionation provided a crude simplification of the sample, and eluent segments were chosen and combined to best represent “low” (roughly <60 kDa), “mid” (roughly 50–100 kDa), and “high” (roughly >100 kDa) molecular weight fractions (Fig. 2a). These resultant fractions were visualized by SDS-PAGE analysis prior to subsequent analyses (Fig. 2b). The breakdown impurity products are clearly present in both the unfractionated and in each of the SEC fractions. Several distinguishable breakdown product bands are present in the SEC prepared fractions, including polypeptides at molecular weights consistent with Fab loss (~100 kDa), Fab fragment (~50 kDa), and free light chain (~23 kDa). In addition to these bands, others are detectable throughout the molecular weight range, including approximately 22–28, 50–60, and 90–110 kDa. The lack of SDS-PAGE mass resolution prevents

the precise number of distinct breakdown components present in each band to be known with any certainty, and each “distinct” band is believed to contain multiple components.

The second, orthogonal separation using reverse-phase chromatography was used to further partition the sample and allow mass spectrometry detection of eluting impurity products. LC-MS analysis of ~200 µg of each SEC fractionation revealed clear chromatographic differences (Fig. 3, left). For example, the deconvoluted masses eluting between 28.0 and 28.5 min (highlighted, left) for unfractionated and each SEC fraction reveal the presence of different molecular weight components, despite identical reverse-phase retention times (Fig. 3, right). Only two ions were detected for the unfractionated analysis, namely 100,145, and 48,326 Da (presumably mAb with one Fab loss and Fab product, respectively). For the “low” SEC fraction, representing those components approximately <60 kDa in mass, two ions were detected (48,305 and 24,445 Da; Fig. 3, middle right). This 24-kDa component detectable only in the “low” SEC fraction is either suppressed by more highly abundant components (*i.e.*, 100 kDa in unfractionated sample) or enriched away (*i.e.*, SEC fractions “mid” and “high”). Additionally, although the ions detected in the “mid” SEC fraction (100,146, and 48,332 Da; Fig. 3, middle right) are seen in the unfractionated analysis, the respective ions eluting within the “high” SEC fraction show a purification of the ~100 kDa component (Fig. 3, bottom right). This example demonstrates the necessary sample simplification that is required for further direct infusion MS/MS sequencing experiments. The entire

Fig. 2. Size-exclusion fractionation of force-degraded antibody sample. Fractions were crudely combined to create fractions of “low” (roughly <60 kDa), “mid” (roughly 50–100 kDa), and “high” (roughly >100 kDa) molecular weight components (a); collected SEC fractions were visualized by SDS-PAGE analysis (b)

Fig. 3. Online LC-MS analysis of each of the collected SEC fractions and unfractionated force-degraded antibody sample (*left*). SEC prepared fractions show a general increase in peak capacity, with more components being detectable in fractionated samples *versus* that of the unfractionated material. Electrospray ionization mass spectra of ions eluting between 28.0 and 28.5 min for each corresponding unfractionated and three SEC fraction (*right*); individual charge state distributions highlighted for each polypeptide present. The SEC fractionation provides the required sample simplification needed for direct infusion, tandem mass spectrometry experiments

intact mass information obtained from each online reverse-phase LC-MS experiment was extracted using the software ProMass for Xcalibur, allowing spectra separated in time to be thoroughly deconvoluted into lists of average, neutral masses. Each SEC fraction was separated in duplicate to allow subsequent MS/MS analysis under both reduced and nonreduced conditions (see below).

Collected reverse-phase fractions from each of the “high”, “mid”, and “low” SEC fractions were subsequently analyzed offline by direct infusion nanoelectrospray ionization mass spectrometry. Because each impurity product could contain intra- or inter-molecular disulfide bonds, every fraction was analyzed separately under reducing and nonreducing conditions to elucidate each impurity structure. Additionally, ions previously detected by online LC-MS were inspected prior to the direct infusion of their respective fraction, and tandem mass spectrometry parameters were chosen as to maximize both confidence in identification and sequence coverage. Roughly, ions <20 kDa were subjected to collisionally induced (CID) and electron transfer dissociation (ETD) fragmentation, whereas ions >20 kDa is mass were subjected CID fragmentation only. Fragmentation data obtained by data-dependant tandem mass spectrometry experiments was directed to ProSightPC v2.0 (ThermoElectron) for sequence assignment. Approximately 10,000

acquired MS/MS spectra were de-isotoped and searched against the known mAb sequence. Searches were filtered based on *E* values (<1e-4) and *P* scores (<1e-4), resulting in ~1,600 confidently identified spectra and ~80 unique linear (*i.e.*, reduced) sequences. Table I and Fig. 2b (band ID) summarizes the major hydrolysis components detected by intact mass detection and MS/MS identification. A complete list of intact masses and MS/MS assignments was established as a database for future reference of the product life cycle monitoring (ELECTRONIC SUPPLEMENTARY MATERIAL (ESM) Table I).

The practicality of this platform for impurity characterization is demonstrated by the separation and mass analysis of several impurity components. As an example, ions derived from the “low” SEC fraction eluting from the reverse-phase separation at ~32 min were detected online by the mass spectrometer. Deconvolution of this detected ion revealed a mass of 47,904.6 Da. Nonreduced analysis by direct infusion mass spectrometry of the respective fraction (time, 32.5–33.0 min) confirmed this deconvoluted mass of 47,902.4 Da (within experimental error); reduced analysis revealed a mixture of two components of mass 24,651.2 and 23,262.7 Da (Fig. 4). MS/MS fragmentation of the reduced sample was able to unambiguously assign the two components to mAb light chain (pyroGlu¹-Cys²¹⁵, 23,262.5 Da) and

Table I. Predominant mAb Breakdown Products Identified by 2D Separation and High Performance Mass Spectrometry

ID #	Observed Nonreduced Mass	Band ID	SEC Fraction(s) Detected	Sequence	Polypeptide Chain	Modification	MS/MS Confirmation
1	11468	a	Mid	pGln1-Gly104	HC	cyclized N-term	Yes
2	11704	a	Low, Mid, High	pGln1-His106	HC	cyclized N-term	Yes
3	11959	a	Low	Glu1-Ala112	LC		Yes
4	13556	b	Low, Mid, High	Lys335-Gly455	HC		Yes
5	13684	b	Mid, High	Lys335-Lys456	HC		Yes
6	15522	c	Mid, High	pGln1-Lys142	HC	cyclized N-term	Yes
7	15855	c	High	pGln1-Gly146	HC	cyclized N-term	Yes
8	23155	d	Low, Mid, High	pGlu1-Glu214	LC	cyclized N-term	Yes
9	23173	d	Low, Mid, High	Glu1-Glu214	LC		Yes
10	23191	d	Low, Mid, High	Glu1-Glu214	LC	C-term cleavage, backbone cleavage, C-term amidation	Yes
11	23356	d	Low, Mid, High	Glu1-Cys215	LC	Cysteinylation	Yes
12	24447		Low	pGln1-Ser228	HC	cyclized N-term	Yes
13	25240	e	Mid	Ser248-Gly455	HC	G1F glycosylation	Yes
14	25395	e	Mid	Gly246-Gly455	HC	G1F glycosylation	Yes
15	46514	f	Mid	Glu1-Cys215	LC	Dimer, cyclized N-term	Partial
16	46551	f	Mid, High	Glu1-Cys215	LC	Dimer	Partial
17	46582	f	Low, Mid, High	Glu1-Cys215	LC	Dimer, +32 Da	Partial
18	46754	f	Low	Gly15-Lys231	HC		No
				Glu1-Cys215	LC		Yes
19	48288 (48151, 48050, 47922, 47807)	f	Low	pGln1-Cys229 through pGln1-His233	HC	cyclized N-term	Yes
				pGlu1-Cys215	LC	cyclized N-term	Yes
20	48306 (48170, 48069, 47939, 47824)	f	Low, Mid	pGln1-Cys229 through pGln1-His233	HC	cyclized N-term	Yes
				Glu1-Cys215	LC		Yes
21	53625	g	Mid	Asp230-Gly455	HC		No
				Thr234-Gly455	HC		No
22	100118	h	Mid, High	Gly15-Gly455	HC		No
				Thr234-Asp443	HC		No
				pGlu1-Cys215	LC		Yes
23	102046	h	Mid, High	pGln1-Gly455	HC	cyclized N-term	No
				Asp230-Gly455/Asp230-Lys456	HC		No
				Glu1-Cys215	LC		Yes
24	102157	h	Mid, High	pGln1-Ser228	HC	cyclized N-term	No
				Asp230-Gly455/Asp230-Lys456	HC	cysteinylation	No
				Glu1-Cys215	LC		Yes

The corresponding identifications (*i.e.*, ID numbers) represents the nonreduced, disulfide bridge-retained breakdown products observed which are comprised of either heavy chain polypeptides only (*e.g.*, ID #1), light chain polypeptides only (*e.g.*, ID #3), or both heavy and light chain polypeptides (*e.g.*, ID #18). Included is the identification number, observed intact (*i.e.*, nonreduced) mass, corresponding band ID (refer to SDS-PAGE gel, Fig. 2b), SEC fractions in which observed, polypeptide sequence(s), polypeptide chain(s), identified modifications, and whether confirmed by MS/MS fragmentation. Refer to ESM Table I for a complete list of identifications

heavy chain fragment (pyroGln¹-Glu²³⁰; mass, 24,651.0 Da). With this information, the impurity component can clearly be assigned to that of a Fab polypeptide, comprised of mAb light chain and N-terminal heavy chain fragment. Interestingly, the MS/MS of mAb light chain component was identified containing a -18.01 Da mass discrepancy, localized to the

cyclization of the N-terminal residue, and consistent with the nonreduced impurity product (*i.e.*, 47,902.4 Da).

To illustrate the ability to parse the heterogeneity of breakdown components, multiple ion species of similar mass were detected by online LC-MS, later identified as various light chain products (see Table I). These ions are each

The ability of this platform to characterize modified breakdown components is demonstrated by the ion species of mass 23,190.7 Da in mass observed eluting chromatographically at 17.5–18.0 min retention. This ion is +17.0 Da

higher than that predicted for a mAb free light chain absent of its C-terminal cysteine residue (theoretical 23,173.5 Da) and is at an approximate 5% relative abundance to all free light chain species (Figs. 5 and 6, top). Further inspection of the reduced fraction analyzed by direct infusion mass spectrometry revealed the presence of several lower molecular weight components (Fig. 6, middle). One component of mass 4,750.3 Da was unambiguously identified as a mAb light chain fragment (Ser¹⁷²-Gln²¹⁴, mass 4751.3 Da) by tandem mass spectrometry (29 b/y fragments identified, *P* score=1e-31). Within the same reduced fraction was detected a complementary ion species of mass 18,444.3 Da in mass, consistent to within 7 ppm of the predicted mAb light chain fragment Glu¹-Asp¹⁷¹. The MS/MS obtained from the respective nonreduced fraction revealed mAb light chain was identified (mass 23,190.7 Da, *P* score=2e-5), also containing a mass discrepancy of +17.0 Da. This discrepancy can be localized to the C-terminal portion of the light chain polypeptide. Taken together, these results confirm the structure of this specific impurity component

XIC- extracted ion chromatogram

Fig. 5. The detection and relative quantitation of three different ion species originating from mAb light chain. Extracted ion chromatograms of species 23,190.6, 23,172.6, and 23,154.5 Da were detected with differing elution times. Inspection of deconvoluted mass spectra reveal clear differences in masses, subsequently fragmented by MS/MS experiments and unambiguously characterized

as that of free light chain containing three modifications, namely (1) C-terminal loss of Cys²¹⁵ residue (−103.0 Da), (2) amidation of resulting C-terminal Glu²¹⁴ residue (−0.98 Da), and (3) backbone cleavage between residues Asp¹⁷¹ and Ser¹⁷² (+18.0 Da), with retention of the intradisulfide bond (Fig. 6, bottom). Only high-resolution intact and tandem mass spectrometry experiments could elucidate such a structure.

DISCUSSION

The field of protein mass spectrometry has advanced considerably over the past few years, now providing the tools which allow characterization of protein therapeutics to a level not previously possible. With the exception of many focused experiments, such as bottom-up LC-MS monitoring of oxidized methionines or deamidations, a careful review of the literature would suggest that few research labs are applying such technologies for the broad characterization of commercial therapeutic mAbs. The term “protein characterization” encompasses many different attributes, including amino acid sequence verification, identification of charge isoforms, a full process- and product-related impurity profile, and glyco-form analysis, to name a few. Many of these can be better understood with the use of mass spectrometry. The goal of this work aims to more accurately and directly characterize the product-related breakdown products of a therapeutic mAb sample without an increase in overall experimental time. As breakdown polypeptides are routinely modified in both predictable and unpredictable ways, either post-translationally *in vivo* or during the degradation process, they must be accounted for in the identification process.

Additionally, application of high-resolution MS and MS/MS can reveal “isobaric” components hidden to low-resolution instruments. High-accuracy intact mass information affords identification of any potential mass discrepancy (*versus* predicted), and high-resolution tandem mass spectrometry experiments facilitate localization of any such modification. This requires only a single system, greatly simplifying the data analysis and interpretation steps in characterization. Lastly, the software tools already exist that reduce the complexity of data and provide a solution for converting 1,000’s of raw full scan and MS/MS fragmentation spectra into reliable information.

The top-down approach provides significant advantages towards the understanding of mAb breakdown products in both information content and throughput over bottom-up peptide mapping strategies. Tertiary structural information related to disulfide bonds, overall isoform composition, and truncations at or near enzymatic cleavage sites are lost when bottom up approaches are employed. For example, ID numbers 5, 6, 18, 23, and 24 (Table I) all have components that end with a C-terminal lysine making them impossible to detect following tryptic digestion. Additionally, the tertiary structural information of ID numbers 15, 16, and 17 (Table I) would have been lost due to reduction of disulfide bonds, which is usually employed in bottom-up experiments prior to enzymatic digestion. Unlike bottom-up approaches which require these additional sample preparation steps (*i.e.*, reduction, alkylation, enzymatic digestion) and LC-MS detection, top-down analysis of breakdown products requires little to no sample preparation for analysis (*i.e.*, reduction and desalting for reduced fractions), thereby allowing for very high throughput. Taken together, this approach is well suited

Observed Sequence (nonreduced):

H₂N-E-I-V-L-T-Q-S-P-G-T-L-S-L-S-P-G-E-R-A-T-L-S-C-X-X-X-X-X-X-X-X-X-W-Y-Q-Q-K-
P-G-Q-A-P-R-L-L-I-Y-X-X-X-X-X-X-X-G-I-P-D-R-F-S-G-S-G-S-G-T-D-F-T-L-T-I-S-R-L-E-P-E-D-
F-A-V-Y-Y-C-X-X-X-X-X-X-X-X-F-G-G-G-T-K-V-E-I-K-R-T-V-A-A-P-S-V-F-I-F-P-P-S-D-E-Q-L-
K-S-G-T-A-S-V-V-C-L-L-N-N-F-Y-P-R-E-A-K-V-Q-W-K-V-D-N-A-L-Q-S-G-N-S-Q-E-S-V-T-E-Q-
D-S-K-D-COOH H₂N-S-T-Y-S-L-S-S-T-L-T-L-S-K-A-D-Y-E-K-H-K-V-Y-
A-C-E-V-T-H-Q-G-L-S-S-P-V-T-K-S-F-N-R-G-E-NH₂

Fig. 6. Identification of free light chain impurity product containing a peptide backbone hydrolysis. Impurity components eluting at ~17.5 min analyzed under nonreducing conditions revealed a tentative identification of free light chain containing a mass discrepancy of +17 Da. Reduced analysis of respective fraction revealed two complementary ions of masses 18,444.3 and 4,749.4 Da. Inspection of acquired MS/MS spectra reveal the presence of disulfide linked cleavage product, containing a C-terminal cleavage (*i.e.*, truncation) and cyclization. The high-resolution intact and MS/MS allows for the detection (and semi-quantitation) of impurity component previously unidentified. Overlaid circles represent theoretical isotopic distributions for each polypeptide

for the routine characterization of therapeutic mAb breakdown impurities.

The breakdown of a monoclonal antibody under stressed conditions follows a general pattern, with additional minor components appearing nonspecifically and product-dependent in nature. This is readily confirmed from inspection of Table I, revealing predictable mAb breakdown components, such as Fab loss, heavy chain hydrolysis, and light chain dimerization. Very little work, however, has been published which provides a means for higher levels of confidence and specificity in breakdown characterization. As can be seen from the data (ESM Table I), multiple components of similar structure are clearly identified. For example, several different

“free” light chain polypeptides species (loss C-terminal cysteine) differing in mass of 18.01 Da are detectable and quantifiable by LC-MS detection (Table I, numbers 8–10; Fig. 5). Interestingly, the structure of the ion species of mass 23,190.6 Da was identified redundantly *via* full-scan analysis of reduced and nonreduced components, and by direct interrogation of the intact (*i.e.*, nonreduced) species by MS/MS fragmentation. Both pieces of information yielded the breakdown component structure as containing a backbone cleavage between residues Asp¹⁷¹ and Ser¹⁷². This improved characterization highlights a real challenge, for example, to the development of biosimilars, in which understanding of the complete product profile (desired molecule and product

impurities) may be required for regulatory approval. Although the rise of biosimilar molecules has yet to be clearly defined, a greater elucidation of “unknown” impurities may likely translate to a higher regulatory hurdle for the safety assessment or product differentiation required for commercialization.

Routine monitoring of therapeutic mAb breakdown components helps to build a database and presents a detailed picture of the molecule where the sites are prone to breakage. This database provides valuable insights into the quality attributes of the molecule of interest. In combination with the bioactivity data of the molecule, critical quality attributes (CQAs) for the molecule can be established. This knowledge of CQAs has a direct impact on quality by design for manufacturing process development and optimization. Specifically, a detailed protein characterization comparability investigation is performed to ensure no additional or previously unseen impurity products arise from such a change which may affect the overall safety or efficacy of the product. Using this platform described here, the quality of process comparability experiments are vastly improved; previously identified breakdown components can be tracked by accurate intact mass and retention times. Without the need for extensive MS/MS fragmentation of known breakdown products, analyses can quickly be performed to detect major abundance changes. Exclusion of ions previously identified by MS/MS can allow mass spectrometry resources to be focused on “unknown” ions. More efficient and informative insights into comparability investigations can help mitigate the risks associated with process changes and can have a major impact upon commercial manufacture of therapeutic drug products.

In addition to breakdown characterization, the platform developed here allows flexibility in implementing complementary mass spectrometry-based protein characterization experiments. The recent native electrospray mass analysis of mAb aggregates, another major product-related impurity, by high-performance TOF instruments demonstrates the increasing ability in applying mass spectrometry to challenging therapeutic characterizations (15). Adopting this analytical framework towards the collection and mass spectrometric analysis of protein aggregates, using both intact mass and MS/MS fragmentation methods may provide insight into the structure and general composition of such impurities. In other applications, the front-end chromatographic separations can be tailored towards experiments specific for the desired molecular attributes. For example, protein charge isoforms can be elucidated by replacing the primary SEC fractionation with ion exchange chromatography (IEC); prepared IEC fractions can be subsequently analyzed by intact (*i.e.*, full-scan MS) and MS/MS experiments, providing top-down structure characterization of charge heterogeneities. These applications will provide deeper insight into the fundamental understanding of therapeutic proteins.

CONCLUSION

This work demonstrates a powerful combination of existing protein mass spectrometry tools for the creation of an analytical platform used for the routine characterization of therapeutic mAb breakdown products. Coupling front-end chromatographic separations together with split-flow LC-MS and fraction collection, a forced-degraded mAb sample was

characterized by high performance intact mass information together with high resolution MS/MS sequencing. Major breakdown products were unambiguously identified and several atypical impurity components reveal a level of characterization not achievable by more traditional analytical technologies. This work provides a direct means towards identifying an impurity profile, and lays the analytical framework for broader application to more in depth characterization of commercial therapeutic protein products.

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