Specific and high-resolution identification of monoclonal antibody fragments detected by capillary electrophoresis–sodium dodecyl sulfate using reversed-phase HPLC with top-down mass spectrometry analysis

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ABSTRACT

In recent years, capillary electrophoresis–sodium dodecyl sulfate (cSDS) has been widely used for high resolution separation and quantification of the fragments and aggregates of monoclonal antibodies (mAbs) to ensure the quality of mAb therapeutics. However, identification of the low-molecular-weight (LMW) and high-molecular-weight (HMW) species detected in cSDS electropherograms has been based primarily on the approximate MWs calculated from standard curves using known MW standards and correlations with fragments and aggregates identified by other methods. It is not easy to collect sufficient amounts of H/LMW species from cSDS for analysis by orthogonal methods and the direct coupling of cSDS with mass spectrometry (MS) is very difficult due to interference from SDS. In this study, we describe the precise identification of H/LMW species detected by cSDS using reversed-phase high performance liquid chromatography (RP-HPLC) coupled with top-down tandem MS analysis. The H/LMW species were first identified by on-line RP-HPLC MS analysis and the RP-HPLC fractions were then analyzed by cSDS to connect the identified H/LMW species with the peaks in the cSDS electropherogram. With this method, 58 unique H/LMW species were identified from an immunoglobulin G1 (IgG1) mAb. The identified fragments ranged from 10 kDa single chain fragments to 130 kDa triple chain fragments, including some with post-translational modifications. This is the first study to clearly identify the antibody fragments, including the exact clipping sites, observed in cSDS electropherograms. The methodology and results presented here should be applicable to most other IgG1 mAbs.

Introduction

Monoclonal antibodies (mAbs) and related biological products are a rapidly growing class of protein therapeutics. Typical mAbs are immunoglobulin G (IgG) molecules composed of two identical heavy chains (HCs) and two identical light chains (LCs) linked by disulfide bonds, with a total molecular weight (MW) of ~150 kDa. MAbs are produced in living cells and protein degradation must be controlled during the production, purification and storage of the drug products. Formation of high-molecular-weight (HMW) protein aggregates and low-molecular-weight (LMW) protein fragments are typical degradation pathways. The HMW species often result from non-covalent associations or photo induced cross linking while the LMW species often result from enzymatic or nonenzymatic (chemical) clipping of the mAb, incomplete formation of disulfide bonds, or scrambling of disulfide bonds.

Accurate identification of the H/LMW species is needed to help understand and control the degradation pathways of mAbs during production and storage. Commonly used techniques for the characterization of size heterogeneity in mAb products include size-exclusion chromatography (SEC), sedimentation velocity analytical ultracentrifugation, and capillary electrophoresis–sodium dodecyl sulfate (cSDS or CE-SDS, also known as capillary gel electrophoresis). Both SEC and cSDS exhibit unique but complementary characteristics for analysis of mAb fragments and aggregates.

As a technique that characterizes size heterogeneity under denaturing conditions, cSDS is superior to the labor-intensive, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method in both sample throughput and resolution. In addition, cSDS employs UV absorbance or fluorescence detection in place of densitometry scans of stained gels for easier and more accurate quantitation. cSDS is often performed under both non-reducing (NR) and reducing conditions to characterize the size heterogeneity of the intact mAb and the intact heavy and light chains, respectively, in the denatured state. This technique has been used for a wide range of applications, including batch release and stability testing, investigation of hinge region clipping, and quantitative analysis of size heterogeneity. However, these studies largely relied on prior knowledge of possible mAb fragments generated under different conditions, estimations of peak MW from the protein standard curve, or correlations with peptide mapping results to identify the cSDS peaks.

In general, the peak assignments have not been confidently verified and it would be difficult to identify unexpected cSDS peaks generated from novel degradation pathways of a mAb...
product. Furthermore, the large uncertainty in MW estimations often prevents accurate and precise peak assignments. An alternative approach is needed for the precise identification of clipping sites and a better understanding of the clipping mechanisms for better control of mAb product quality. Furthermore, compiling the results for many mAbs will allow some standard cSDS peaks to be quickly identified in all mAb products.

Mass spectrometry (MS) is an ideal tool for precise protein sequence identification. SEC coupled with MS has been used to identify several mAb-related species under non-denaturing conditions, but the resolution of commonly used SEC is not sufficient to identify many of the fragments detected by cSDS. In addition, fragments often bind non-covalently to intact mAb or HMW species and remain inseparable on SEC. In theory, cSDS coupled to MS could fulfill the requirement of both separation and MS detection, but the matrix components of cSDS interfere with on-line electrospray ionization-MS and preparative-scale cSDS has not been developed for size variant collection and offline MS characterization. As a MS-friendly separation technique based on molecule hydrophobicity, reversed phase-high performance liquid chromatography (RP-HPLC) is well known for its high resolution separation, and has been widely coupled with quadrupole time-of-flight (Q-TOF) or orbitrap mass spectrometers for high resolution MS analysis of intact mAbs, mAb subunits and mAb fragments.

In this study, we directly identified dozens of fragments of a typical IgG1 mAb by RP-HPLC on-line coupled with intact mass and top-down higher energy collisional dissociation (HCD) tandem MS (MS/MS). Some fragments with unexpected post-translational modifications (PTMs), such as intact LC disulfide-linked with glutathione and half IgG containing thioether, were also identified in the process. RP-HPLC fractions containing the identified H/LMW species were then analyzed by cSDS to assign each identified species to a peak in the cSDS electropherogram. The workflow we used is shown in Scheme 1. The results of this study provided precise identification of most fragment peaks in the cSDS electropherogram and the associated clipping sites, which can now be used to better understand the degradation mechanisms and develop potential controls for the cleavage of mAbs.

Results

NR cSDS analysis of mAbs

Unstressed and heat-stressed (HS) mAbs were first analyzed by cSDS under non-reducing conditions. As shown in Figure 1, a total of 10 H/LMW species were visible in a zoom view of the electropherogram for the unstressed mAb, but were present at very low levels. The purity of unstressed mAb measured by NR cSDS was 96.1% (intact IgG), with 0.2% HMW species (peak NR10) that were larger than intact IgG and 3.7% LMW species (peaks NR1-9) that were smaller than intact IgG. HS mAb contained the same 10 H/LMW peaks (NR1-10) that were visible in the unstressed sample, as well as 3 new LMW peaks (NRB, NRC and NRD) that were not visible in the unstressed sample. All 13 peaks were present at higher levels in the HS sample, although the levels of NR2, NRB, NRC and NRD in the HS mAb sample appeared to increase more than the other peaks.

RP-HPLC MS identification of the fragments in NR mAb

The LMW species in NR, HS mAb were identified using RP-HPLC Q-TOF MS analysis by matching the measured MW with the theoretical MW of predicted HC or LC fragments. The RP-HPLC method was optimized by screening columns from different vendors (Waters vs. Agilent) with different resins (C8 vs. C4 vs. diphenyl) and different porosities (core-shell type vs. totally porous), different elution temperatures, and different elution buffers (trifluoroacetic acid vs. formic acid, with and without added isopropanol). We also extended the gradient in certain regions to improve peak resolution, especially for those species eluting close to the intact IgG. With this optimized RP-HPLC method, over 30 unique LMW fragments were identified in the HS mAb as labeled in Figure 2 and listed in Table S1, including 17 fragments that were also present in the unstressed mAb. The smaller fragments below 40 kDa typically included the N-terminus or the C-terminus of the HC or LC. The larger fragments over 40 kDa were typically LC and HC fragments linked by inter-chain disulfide bond(s) to either intact or fragmented HCs and LCs.
As displayed in Scheme 1, the NR fragments identified by MS were collected off-line and analyzed by NR cSDS to connect the identified fragments with peaks in the cSDS electropherogram. The NR cSDS electropherograms for 8 of the collected RP-HPLC fractions (panels A-H) and the original (unfractionated) HS mAb (panel I) are presented in Figure 3. Typically, the most abundant peak in the cSDS electropherograms for the RP-HPLC fractions co-migrated with a peak in the cSDS electropherogram for the unfractionated mAb sample, which allowed the cSDS peaks to be identified. For example, IgG-Fab3 was the major component eluting near 30.9 min on RP-HPLC (Figure 2) according to MS analysis. cSDS analysis of the RP-HPLC fraction collected at this time showed a major cSDS peak that co-migrated with peak NR8 at 25.4 min in the electropherogram (compare panels G and I in Figure 3). In this way, fragment IgG-Fab3 was assigned to NR cSDS peak NR8. In some cases, the RP-HPLC fraction contained multiple fragments that co-migrated with different cSDS peaks. These fragments were assigned to the corresponding cSDS peaks according to the ranking of their MWs and electrophoretic migration time. For example, the half IgG, IgG-Fab1 and IgG- HC (1–212) fragments eluting from 31.7–32.6 min on RP-HPLC (Figure 2) were assigned to cSDS peaks NR7, NR8 and NR9, respectively, in Figure 3(h).

A labeled, NR cSDS electropherogram based on this comprehensive analysis is shown in Figure 4. All the mAb fragments identified by MS could be assigned to one of the LMW peaks in the NR cSDS electropherogram, but none of the RP-HPLC fractions contained a species that could be assigned to cSDS peak NR10, which was larger than the intact IgG. Results from an in-gel tryptic digestion of the HMW band in an SDS-PAGE gel followed by peptide mapping suggested that NR10 was likely an IgG dimer (data not shown).

Figure 1. NR cSDS Analysis of mAb. Zoom views of the electropherograms from the unstressed (top trace) and HS (bottom trace) mAb are presented. The H/LMW peaks are labeled in a sequential order.

Figure 2. RP-HPLC MS Analysis of HS mAb. A full view of the RP-HPLC UV chromatogram is presented in panel A and a zoom view of the 13–29 min region is presented in panel B. The peaks identified by MS are labeled. Fragments with PTMs are marked with different icons: ▽ = Oxidation (+16 Da), △ = hydrolysis of amide bond (+18 Da), and ϕ = IsoAsp.

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Reduced cSDS analyses of mAb

Unstressed and HS mAb were also analyzed by cSDS under reducing conditions. Two major peaks corresponding in MW to intact HC and LC were visible in the full view of the reduced cSDS electropherogram of unstressed mAb (data not shown). A total of 11 H/LMW species were visible in a zoom view of the electropherogram for the unstressed mAb sample (Figure S1, top trace), but were present at very low levels. The purity of unstressed mAb measured by reduced cSDS was 96.7% (HC and LC), with 1.9% HMW species that were larger than intact HC and 1.4% LMW species that were smaller than intact HC.

As shown in Figure S1, HS mAb contained the same 11 H/LMW peaks (R1-10 and AgHC) that were visible in the unstressed sample, as well as 4 new LMW peaks (RB, RC, RD, and RF) that were not visible in the unstressed mAb. Most of these H/LMW peaks were present at higher levels in the HS mAb, although peaks RD, R4, R5 and R6 appeared to increase more in the heat stressed mAb than the other peaks.

RP-HPLC MS identification of the fragments in reduced mAb

A total of 48 H/LMW species (not including species with PTMs) were identified in the reduced HS mAb by RP-HPLC Q-TOF MS analysis, including 45 LMW fragments, 1 aglycosylated HC (AgHC), and 2 HMW fragments. These fragments were labeled in Figure S2 and listed in Table S2, including 27 that were also present in the unstressed mAb.

As shown in Figure S1, the reduced, HS mAb fragments identified by MS were collected off-line and analyzed by reduced cSDS to connect their identity with the peaks in the cSDS electropherogram. The reduced cSDS electropherograms for 9 of the RP-HPLC fractions (panels A-I)
and the original (unfractionated) HS mAb (panel J) are presented in Figure S3. Assignment of the AgHC peak was confirmed by reduced cSDS of PNGase-treated mAb (Figure S4).

A labeled, reduced cSDS electropherogram based on this comprehensive analysis is presented in Figure 5. Most of the mAb fragments identified by MS could be assigned to one of the H/LMW peaks in the reduced cSDS electropherogram. Peaks R7 and R10 were not identified by RP-HPLC MS. However, the two peaks both migrated to an earlier time after PNGase F treatment (compare a and b in Figure S4B), suggesting that R7 and R8 were N-glycosylated species. Based on the migration time of the two species, the MW for R7 and R10 could be estimated at 60 kDa and 110–120 kDa, respectively.

**Confirmation of H/LMW fragments by top-down HCD MS/MS**

Top-down RP-HPLC HCD MS/MS analysis was performed to verify the N- or C-terminal sequences of the intact fragments identified by Q-TOF intact mass analysis. For example, IgG-Fab3 contains an intact LC, an intact HC and a HC fragment, and is one of the largest fragments in the NR cSDS electropherogram. As shown in Figure S5B, three groups of b ions confirmed the N-terminal sequences of intact LC, intact HC, and HC 222–446, respectively, and a series of y ions confirmed the identical C-terminal sequences from intact HC and HC 222–446.

MS/MS analysis also revealed some fragments with unexpected modifications. As shown in Figure 6(a), two LMW fragments (22,846 Da and 23,033 Da) detected in the NR mAb by intact mass analysis had slightly larger masses than

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**Figure 4.** Labeled NR cSDS electropherograms for the unstressed (a) and HS (b) mAb. Peaks were identified by HPLC-MS analysis followed by NR cSDS analysis of the HPLC fractions.

**Figure 5.** Labeled reduced cSDS electropherograms for the unstressed (a) and HS (b) mAb. Peaks were identified by HPLC-MS analysis followed by reduced cSDS analysis of the HPLC fractions.
intact LC (22,727 Da), but their MWs did not match any portion of the HC or any multi-chain fragments deduced from the unmodified mAb sequence. HCD orbitrap MS$^2$ successfully identified the two fragments as intact LCs disulfide-linked with free cysteine (Figure 6(b)) or with glutathione (data not shown) on Cys216 near the LC C-terminus.

Several modified H/LMW fragments were also present in the reduced mAb. For example, a HMW fragment that was 32 Da smaller than normal half IgG was identified from reduced HS mAb by top-down HCD MS$^2$ (Figure S6). This fragment was a half IgG containing a non-reducible inter-chain thioether bond that linked intact HC with intact LC. The non-reducible inter-chain thioether was further confirmed by Cys-alkylation of the fully reduced HS mAb (Figure S7). In another example, modified LC fragments or intact LC from HS mAb (marked with ϕ in Figure 2 and Figure S2) were observed to elute slightly before the unmodified species on the RP-HPLC chromatogram. The modified species all contained isoAsp95, and shared the same MW and nearly identical top-down HCD MS$^2$ spectra as their unmodified forms (data not shown). The abundance of the isoAsp form of these fragments also increased upon heat stress. This was consistent with peptide mapping results, which showed that nearly 50% of LC Asp95 for this mAb was converted to isoAsp after HS (data not shown).

Identified mAb clipping sites

As summarized in Table 1 and shown visually in Figure 7, a total of 36 clipping sites were identified in the HS mAb. Importantly, 17 of these clipping sites also occurred in the unstressed mAb (labeled with * in Table 1). Most clipping sites could be identified by analysis of the NR mAb, but some could only be identified or confirmed by analysis of the reduced mAb.

Ideally, clipping of a mAb should generate two fragments that both can be identified by RP-HPLC MS analysis of the non-reduced mAb sample, but very often only the smaller fragment was identified, while the larger, sister fragment was not detectable by MS due to co-elution with other large fragments or intact IgG. The larger fragments, however, could further dissociate in the reduced condition and be detected, which often allowed confirmation of the clipping sites. For example, the clipping between LC Asp95-Gly96 should generate a N-terminal LC 1–95 fragment and the IgG minus LC1–95 sister fragment, but only LC 1–95 was detected by RP-HPLC MS in the NR mAb (Table 1). However, reduction of the mAb dissociated the disulfide-linked, IgG minus LC1-95 sister fragment, which allowed MS detection of the C-terminal, LC 96–217 fragment. This additional information corroborated the identification of the clipping site.

Some clipping sites we identified in the NR sample were unexpected. For example, clipping between HC Asp270-Pro271 generated fragments HC 1–270 and HC 271–446 that were still linked together by the Cys261-SS-Cys321 disulfide bond within the CH2 domain (Figure 7). Clipping at this site generated an IgG species that was 18 Da heavier than the native IgG due to hydrolysis of the amide bond between HC Asp270-Pro271. These clipped, but non-dissociated, species are normally not detected due to their co-elution with the intact IgG by cSDS, RP-HPLC, or SEC, and are almost indistinguishable from oxidized IgG by MS. Both HC 1–270 and HC 271–446, however, were identified in the reduced HS mAb (50.5 min and 27.1 min, respectively, in Figure S2). Knowing these species are present in reduced mAbs is important because they may have compromised bioactivity due to altered conformations. Thus, identifying these additional clipping sites can help reveal additional degradation pathways and potentially improve the control of product quality.

Discussion

A total of 58 unique, product-related fragments were identified in NR and reduced preparations of an IgG1 mAb using a combination of RP-HPLC MS and cSDS. These fragments
were likely generated by chemical or enzymatic hydrolysis of the peptide backbone or disruption of disulfide bonds. To the best of our knowledge, this is the first time that such a large number of IgG fragments detected in cSDS were precisely identified by MS analysis. There were also several HMW cSDS peaks (NR10, R7 and R10) not identified directly by RP-HPLC MS. These species were either not resolved from high abundant species by RP-HPLC or were non-covalent complexes that underwent dissociation in the RP-HPLC condition.

Some of the identified fragments or clipping sites were also detected in other IgG mAbs. This is not surprising since the amino acid sequence is so well conserved. As shown in Figure S9 A and B, some of the same NR fragments were also identified in a second IgG1 mAb, such as the commonly known fragments Fab, IgG-Fab and half IgG, as well as some fragments that were not well known before, such as HC1–214, IgG–HC(1-214), HC1-139, and HC252-448 (compare Figure S9 A and B). Some common fragments were also detected in the reduced cSDS electropherogram (compare Figure S10 A and B). Thus, the cSDS peak assignments provided in this study should be a good guide for all IgG1 mAbs and this type of exhaustive characterization will not be necessary for other mAbs. However, some clipping sites and the resulting fragments were not detected in all mAbs. For example, LC dimer, LC1-95 and LC96-217 were detected only for the mAb in Figure S9A and Figure S10A, but not for the second mAb in Figure S9B and Figure S10B. Thus, the approaches described in this study can be used in selected circumstances to identify new peaks as part of investigations.

Although many of the fragments identified in this study were present at very low levels (especially for the unstressed mAb), and thus unlikely to affect the safety or efficacy of this product, some of these fragments could exist at higher levels if the degradation pathways are not properly controlled. Multiple factors could induce different clipping pathways resulting in these different fragments, including but not limited to the pH and composition of the formulation buffer, the primary amino acid sequence in the CDRs, and differences in the higher order structures. The comprehensive list of clipping sites provided in this study should form a foundation for the analysis of antibody fragmentation and a better understanding of these different degradation pathways.

Among the identified H/LMW species were several fragments with PTMs, such as LC-SS-glutathione and LC-SS-cysteine. Glutathione is a tripeptide that is present in almost all eukaryotic cells. This thiol compound is widely used as a medium supplement in biomanufacturing and is very important for cell growth and viability. It is therefore not surprising to detect LC-SS-glutathione fragments in a mAb sample. As a tripeptide, glutathione may degrade into cysteine, which may bind to a LC by disulfide bond to form LC-SS-cysteine. As the glutathione and

| Table 1. Summary of the Identified Clipping Sites in the mAb. |
|---------------------------------------------------------------|
| **Chain** | **Clipping region** | **Clipping site** | **Fragment from NR mAb** | **Fragment from reduced mAb** |
| HC Domain Interface | Ala118-Ser119 | HC 1–118 | HC 1–118 |
| | Lys133-Ser134* | HC 1–133* & IgG – HC (1–133) * | HC 1–133* & HC 134–446* |
| | Thr135-Ser136 | HC 1–135 | HC 1–135 & HC 136–446 |
| | Ser136-Gly137 | HC 1–136 | HC 1–136 |
| | Gly137-Gly138* | HC 1–137* | HC 1–137* & HC 138–446* |
| | Asp212-Lys213* | HC 1–212* & IgG – HC (1–212) * | HC 1–212* & HC 213–446* |
| | Lys213-Lys214 | N/D | HC 1–213 |
| | Lys218-Ser219 | HC 1–218 | HC 1–218 |
| | Ser219-Cys220 | HC 1–219 | HC 1–219 |
| | Asp249-Thr250* | IgG – HC (250–446) * & HC 250–446 | HC 1–249* & HC 250–446* |
| | Asn325-Lys326 | IgG – HC (250–446) | HC 326–446 |
| | Asp356-Glu357* | IgG – HC (250–446) * & HC 250–446 | HC 326–446 |
| | Val188-Pro189 | N/A | HC 189–446 |
| | Asp270-Pro271* | N/A | HC 270–446 & HC 271–446* |
| | His285-Asn286 | N/A | HC 286–446 |
| | Asn286-Ala287 | N/A | HC 287–446 |
| | Asn297-Ser298 | N/A | HC 298–446 |
| | Asn315-Gly316 | N/A | HC 316–446 |
| Hinge Region | Cys220-Asp221 | Fab (LC + HC (1–220)) | HC 1–220 & HC 221–446 |
| | Asp221-Lys222* | Fab (LC + HC (1–221)) * & IgG – Fab | HC 1–221* & HC 222–446* |
| | Lys222-Thr223 | IgG – Fab | HC 222–446 |
| | His224-Thr225 | Fab (LC + HC (1–224)) & IgG – Fab1 | HC 1–224 & HC 225–446 |
| | Ala231-Pro232 | N/D | HC 226–446 |
| | Leu235-Gly236 | IgG – HC (236–446) & HC 237–446 | HC 237–446 |
| | Gly236-Gly237 | IgG – HC (236–446) & HC 237–446 | HC 238–446 |
| | Gly237-Pro238 | IgG – HC (237–446) & HC 238–446 | HC 238–446 |
| LC Domain Interface | Glu1-Asp* | LC 2–217 | LC 2–217 |
| | Gln6-Pro7* | LC 2–217 | LC 2–217 |
| | Glu215-Cys216* | LC 1–215* | LC 1–215* |
| | Cys216-Ser217* | LC 1–216* | LC 1–216* |
| | Asp95-Gly96* | LC 1–95* | LC 1–95* & LC 96–217* |
| | Gly96-Leu97* | LC 1–96* | LC 1–96* & LC 97–217* |
| | Leu97-Ser98 | LC 1–97 | LC 1–97 & LC 98–217 |
| | HC-5-5-HC | Cys226-S-S-Cys226* | Half IgG | N/A |
| | | Cys228-S-S-Cys228* | Half IgG | N/A |
| | | Cys220 (HC) -5-5 -Cys216 (LC)* | LC+Cys* & LC dimer | N/A |

*: Clippings/fragments also observed in the unstressed mAb
N/D: not detected
N/A: should not exist in the NR or reduced mAb
free cysteine should have been removed from the mAb product during downstream purification, the level of the two species should not increase after manufacturing. This is consistent with results presented in Figure 4, in which the level of the corresponding cSDS peak at 16.3 min did not increase after three months of HS, suggesting the low risk of this modification during sample storage. Another example is half IgG with an inter-chain thioether linkage. Formation of thioether disulfide bonds has been observed in IgG1 antibodies, with increasing levels upon storage.

This is consistent with results presented in Figure 5, in which the corresponding cSDS peak at 23.3 min increased by ~50% after three months of HS.

An additional highlight of this work was direct verification of the N- or C-terminal sequences for most fragments by top-down MS/MS. Although intact mass measurement allows the prediction of possible sequences for most IgG fragments, the risk of incorrect identification of specific cleavage sites may still exist in some circumstances. For example, (1) unexpected PTMs could produce mass shifts for a fragment; (2) more than one fragment could share the same or similar MW, especially when intra-chain disulfide bonds are incompletely reduced; (3) multiple clippings could create two or more truncated polypeptides linked by disulfide bond(s); and (4) MS accuracy drops as protein MW increases, which increases the uncertainty in assigning the larger fragments. Some of the above concerns were addressed successfully in this study by top-down, on-line RP-HPLC MS/MS, including the identification of unexpected MW increase on intact LC due to disulfide-linkage with free cysteine and glutathione, and the identification of an unexpected MW decrease on half IgG due to formation of a thioether bond. Top-down MS/MS identification of intact fragments is more conclusive and convenient than traditional approaches using peptide map analysis of chromatography fractions or SDS-PAGE bands. Several studies have recently used top-down, middle-down or middle-up MS/MS approaches to map the complete primary structure of a mAb, but these approaches have rarely been used to identify low level mAb fragments. Wang et al. reported RP-HPLC HCD MS/MS characterization of the disulfide-reduced forms of an anti-C. difficile mAb and 7 product-related fragments (10–39 kDa MWs) fractionated by cation-exchange chromatography. Our study was more comprehensive than previous studies on the identification of mAb fragments, and connected the fragments sequences identified by RP-HPLC MS analysis with the quantifiable H/LMW species peaks detected by cSDS.

Direct coupling of SEC to native MS has been reported for the characterization of mAb fragments in non-denatured state. By taking advantage of the MS-friendly elution buffer (ammonium acetate), the on-line SEC-MS method avoids offline fractionation and can be used to directly identify protein fragments and aggregates based on intact mass. The product-

![Figure 7. IgG1 structure showing the identified clipping sites (C-terminal side of labeled amino acid residues) along the backbone sequence of mAb. The solid X with longer size indicates the clipping site identified from both unstressed and HS mAb; the open X with shorter size indicates the clipping site identified from only HS mAb; the red X indicates the clipping site located within CDR; the green X indicates the clipping site located within non-CDR.](image-url)
related species detected by SEC-MS, which are typically IgG multimer, IgG dimmer, IgG-Fab, Fab, LC, as well as some non-covalent complexes of these subunits,\textsuperscript{6} can be identified without the extra steps for peak assignment as presented in our study. However, SEC is a low-resolution technique that cannot be used to identify many of the IgG fragments present in purified mAb preparations. Generally, the large molecules need to differ by at least 2-fold in MW to be resolved.\textsuperscript{32}

Furthermore, many of the structural details obtained by RP-HPLC MS analysis cannot be obtained by online SEC-MS analysis. One limitation is that SEC-MS mainly detects native mAb complexes with small charge-state envelope and condensed ion signals, which are suitable for intact mass analysis to determine the MW information, but not appropriate for top-down MS/MS to identify unexpected PTMs.\textsuperscript{16} For example, LC-SS-cysteine and LC-SS-glutathione were both identified directly by top-down on-line RP-HPLC MS/MS in this study, but, as reported by Haberger et al.,\textsuperscript{3} they were detected by SEC-MS as a LC-SS-cysteine/LC-SS-glutathione heterodimer non-covalently associated with a ½ mAb monomer. In that report, the entire complex had to be isolated off-line from SEC, followed by Lys-C digestion and peptide mapping to identify the LC modifications.

SEC also cannot be used to identify protein fragments that are held together by covalent and non-covalent interactions. For example, some fragments such as HC 1–270 and HC 271–446 were detected under reduced conditions in this study, but were not detected by SEC-MS under non-denaturing and non-reducing conditions. Many of these fragments were generated by clipping within the C\textsubscript{\text{H}2} domain and were likely held together by covalent and non-covalent interactions, which prevented their identification by SEC-MS. Thus, RP-HPLC MS analysis is able to identify many more antibody fragments than SEC-MS and provides a complement to the published literature on this topic.

It is worth noting that fragments with increased migration time by cSDS did not always correlate with an increased MW even though they were denatured by SDS treatment (Table S3 and Figure S8). For example, the Fab1 fragment with a MW of 46.9 kDa migrated at 18.9 min (Figure 3(e)), while the LC dimer with a slightly lower MW (45.5 kDa) migrated 0.5 min later (Figure 3(f)). Since LC dimer and Fab both have an intact LC, the migration time difference was probably due to the different cross-sectional area of the second chain (i.e., LC vs. HC 1–220/1–221/1–222/1–224). A similar discrepancy was observed for a group of LC fragments (LC 1–215, LC-SS-cysteine, LC-SS-glutathione, and their isoAsp isoforms) and a group of HC fragments (HC 1–212, 1–218 and 1–219), as shown in Figure S8 A-G. These LC fragments have slightly smaller MWs than the HC fragments, but migrated ~1 min slower than the HC fragments in cSDS. Although the V\textsubscript{\text{L}-C\textsubscript{L}} domain has a slightly lower MW than the V\textsubscript{\text{H}-C\textsubscript{H}1} domain, the V\textsubscript{\text{L}-C\textsubscript{L}} domain tends to slow down the migration of fragments more effectively compared to the V\textsubscript{\text{H}-C\textsubscript{H}1} domain (Table S3). These results indicate that peak assignments based solely on the fragment MW and the migration time in the cSDS electropherogram may not be correct.

A direct outcome of this study was the precise identification of mAb clipping sites. A complete map of the mAb clipping sites identified in this study is presented in Figure 7. Similar to the results presented in previous studies,\textsuperscript{7,9,25,33} peptide bonds near the hinge regions and domain interfaces were very susceptible to hydrolysis (Table 1). For example, 8 of the 36 clipping sites were located in the hinge region, including 4 in the upper hinge region (HC Cys220-Asp221, Asp221-Lys222, Lys222-Thr223 and His224-Thr225), and 4 in the lower hinge region (HC Ala231-Pro232, Leu235-Gly236, Gly236-Gly237, Gly237-Pro238). Of the 36 clipping sites, 16 were located in regions that connect two domains, including the V\textsubscript{\text{H}-C\textsubscript{H}1}, hinge region-C\textsubscript{\text{H}2}, C\textsubscript{\text{H}2}-C\textsubscript{\text{L}1}, V\textsubscript{\text{L}-C\textsubscript{L}} interfaces, as well as in the region near LC-SS-HC inter-chain disulfide bond. Nine of the 36 clipping sites were located within a defined structural domain of the mAb. The fragments generated by clipping in the C\textsubscript{\text{H}2} domain at HC Asp270-Pro271, His285-Asn286, Asn286-Ala287, Asn297-Ser298 and Asn315-Gly316 and in the C\textsubscript{\text{H}1} domain at HC Val188-Pro189 were connected by disulfide bonds (Figure 7), and could only be identified in the reduced mAb sample (Table 1). In addition to the clipping of amide bonds within the polypeptide backbone, disruption of disulfide bonds also occurred,\textsuperscript{7} as evidenced by the fragments half IgG, LC dimer, LC-SS-cysteine, and LC-SS-glutathione detected in the NR mAb.

Previous studies demonstrated that some sequence motifs such as Asp-Xxx, Xxx-Asp, Gly-Gly, Gly-Leu, Ser-Gly, Leu-Gly, Asn-Ser, Ala-Ser, Leu-Ser, and Xxx-Cys are more susceptible to cleavage than others, and fragmentation at the C-terminus of Asp residues has been identified as a major degradation pathway of mAbs under mildly acidic conditions.\textsuperscript{3} These conclusions are consistent with many of the clipping sites identified in our study (Table 1). Of the 14 peptide bond cleavage sites found in both unstressed and HS mAb (starred sites in Table 1), 6 sites matched the Asp-Xxx sequence motifs.

Identification of clipping sites will help us better understand mAb degradation pathways and develop better methods to control clipping during the manufacture and storage of mAb products. To help visualize the location of these clipping sites, a full-length structure of the mAb is displayed as a ribbon diagram with zoom views of 8 major clipping sites in 3 regions of the protein (Figure 8). Fragments generated by clipping at these 8 major sites were detected in the unstressed mAb and the levels of these fragments increased after HS. Clipping sites 1, 2, 4, 7 and 8 are located in flexible loops with high solvent exposure. Clipping sites 3, 5 and 6 are located within β-strand or α-helical secondary structures, but they are close to flexible loops and occur at the C-terminus of Asp residues. Importantly, site 8 (LC Asp95-Gly96) is located in the LC CDR-3, which is involved in antigen binding, and site 5 (HC Asp249-Thr250) is located within the FcRn binding site. Clippings at these two sites could affect the biological function of this mAb.

In summary, the methodology presented in this study can be used for either comprehensive cSDS peak identification (suitable for large therapeutic molecules with novel structures), or as an investigational tool for the identification of one or several cSDS peaks of interest. The latter would only require RP-HPLC MS identification of the fragment(s) that fall into the MW range of the target cSDS peaks, followed by re-injection of one or several off-line RP-HPLC fractions of the identified fragments for cSDS analysis to match the migration time of the target cSDS peak(s). If any of these H/LMW species were later identified as critical quality attributes, their
structural information, including protein sequence and modifications, could provide valuable feedback to the manufacturing or storage conditions.

Materials and methods

Materials and reagents

Reagents used for sample preparation and HPLC analysis included DL-dithiothreitol (DTT) (Sigma), 2-mercaptoethanol (Sigma), N-ethylmaleimide (Research Organics), PNGase F (Prozyme), 1 M tris-hydrochloride buffer pH 7.5 (Corning), trifluoroacetic acid (Thermo Scientific), acetonitrile (Honeywell), and in-house prepared Milli-Q Water.

Materials used for cSDS analysis were provided in the Beckman Coulter SDS-MW Analysis Kit (PN 390953), including capillary (50 μm I.D. bare-fused silica), SDS-MW gel buffer (proprietary formulation, pH 8, 0.2% SDS), SDS-MW sample buffer (100 mM Tris-HCl, pH 9.0, 1% SDS), 10 kDa internal standard (I.S., 5mg/mL), acidic wash solution (0.1 N HCl), and basic wash solution (0.1 N NaOH).

The unstressed mAb sample was a full-length human glycosylated IgG1 produced in house in CHO cells.

Sample preparation

Unstressed mAb was formulated at 100 mg/mL in 10 mM L-histidine/L-histidine hydrochloride (pH 5.8), 8.5% (w/v) sucrose, and 0.055% (w/v) polysorbate 80. Heat-stressed (HS) mAb was generated by incubation in a sealed glass vial at 40°C for 3 months.

To reduce the disulfide bonds for HPLC-MS analysis, 100 mg/mL mAb was diluted to 10 mg/mL by mixing with deionized water and 1 M DTT at 1/8/1 (v/v/v) ratio and incubated at 37°C for 30 min.

Aglycosylated mAb was generated by mixing 10 μL of 100 mg/mL unstressed mAb with 90 μL of 100 mM tris-HCl (pH 7.5) buffer and 20 μL of PNGase F (2 U/mL), followed by incubation at 37°C for 24 hrs. The sample was then diluted with 10 μL of 1 M DTT and incubated at 37°C for 30 min to selectively reduce inter-chain disulfide bonds.

All the samples were stored at −70°C prior to analysis.

RP-HPLC and MS

Samples were analyzed by RP-HPLC using an Agilent Technologies 1100 Series HPLC system with an on-line UV detector coupled to a Waters Micromass Q-TOF Ultima API-US mass spectrometer. A 50 μg aliquot of mAb was loaded onto an Agilent AdvanceBio RP-mAb C4 column (2.1 × 150 mm, 3.5 μm) and eluted with a gradient of acetonitrile in 0.05% (v/v) trifluoroacetic acid at 80°C with a flow rate of 0.3 mL/min. Mobile phase A was 0.05% trifluoroacetic acid in water (v/v), and mobile phase B was 0.05% trifluoroacetic acid in 80% acetonitrile. NR mAb samples were eluted using the following gradient: 25% B for 5 min, 25–35% B for 5 min, 35–42.5% B for 15 min, 42.5–44% B for 15 min, 44–100% B for 2 min, 100–25% B for 2 min, and 25% B for 8 min. Reduced mAb samples were eluted using the following gradient: 25% B for 5 min, 25–41% B for 24 min, 41–43.5% B for 25 min, 43.5–46% B for 6 min, 46–100% B for 2 min, 100–25% B for 1 min, and 25% B for 12 min.

For Q-TOF MS analysis, samples were scanned from m/z 800–4000 with 1 s scan duration and 0.1 s inter-scan delay. The full MS signal across the total ion chromatogram peak of each fragment was averaged and deconvoluted to MW (Da) by MaxEnt I (Waters).

Top-down RP-HPLC HCD MS/MS analysis of the mAb fragments was performed using an Agilent Technologies 1290 Infinity LC system with a Thermo Scientific Q Exactive hybrid quadrupole-orbitrap mass spectrometer. A more detailed description of the HCD MS/MS analysis is provided in the Supplemental Information.

Off-line RP-HPLC fractionation

Aliquots (50–300 μg) of HS mAb (NR or reduced) were loaded onto an Agilent AdvanceBio RP-mAb C4 column and eluted with the same gradients used for HPLC Q-TOF MS analysis. MAb fragments eluting from the column were monitored in real time by UV absorption and collected into different fractions. Fractions collected from multiple runs were pooled to accumulate enough material for cSDS analysis of each fraction. The combined fractions were dried using a SpeedVac concentrator and stored at −70°C prior to cSDS analysis.

Figure 8. Structural model showing the major cleavage sites in the mAb.
cSDS analysis

The unstressed and HS mAb samples were diluted to 10 mg/mL with water before cSDS analysis. The dried RP-HPLC fractions collected from the NR and reduced HS mAb samples were reconstituted in 15 µL of water.

For NR cSDS analysis, 30 µL of each mAb sample was diluted with 156 µL of 25 mM bis-tris/citrate buffer (pH 7.0) in 1% SDS, 4 µL of 5 mg/mL 10 kDa I.S., and 10 µL of 125 mM N-ethylmaleimide. Each reconstituted HPLC fraction was diluted with 78 µL of 25 mM bis-tris/citrate buffer (pH 7.0) in 1% SDS, 2 µL of 5 mg/mL 10 kDa I.S., and 5 µL of 125 mM N-ethylmaleimide. The diluted samples were incubated at 70°C for 5 min and then cooled to room temperature.

For reduced cSDS analysis, 30 µL of each mAb sample was diluted with 161 µL of SDS-MW sample buffer, 4 µL of 5 mg/mL 10 kDa I.S., and 5 µL of 20% (v/v) 2-mercaptoethanol (diluted in SDS-MW sample buffer). Each reconstituted RP-HPLC fraction was diluted with 80.5 µL of SDS-MW sample buffer, 2 µL of 5 mg/mL 10 kDa I.S., and 2.5 µL of 20% (v/v) 2-mercaptoethanol. The diluted samples were incubated at 70°C for 5 min and then cooled to room temperature.

NR and reduced cSDS were performed using the same electrophoresis procedure on a Beckman Coulter ProteomeLab™ PA 800 Protein Characterization System equipped with photo diode array detection. Each sample was injected into the capillary for 20 seconds at 5 kV (reverse polarity), with a 10 µL injection volume, followed by separation at 15 kV (reverse polarity) for 35 min in the capillary containing SDS-MW gel buffer.

Abbreviations

mAb Monoclonal antibody
IgG1 immunoglobulin G1
HC heavy chain
LC light chain
H/LMW high-/low-molecular-weight
PTM post-translational modification
HS heat-stressed
cSDS capillary electrophoresis–sodium dodecyl sulfate
RP-HPLC reversed-phase high performance liquid chromatography
SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC size-exclusion chromatography
NR non-reducing
MS mass spectrometry
Q-TOF quadrupole time-of-flight
MS/MS tandem MS
HCD higher energy collisional dissociation

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