Comparison of SEC and CE-SDS methods for monitoring hinge fragmentation in IgG1 monoclonal antibodies

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ABSTRACT

Fragmentation of monoclonal antibodies is a critical quality attribute routinely monitored to assess the purity and integrity of the product from development to commercialization. Cleavage in the upper hinge region of IgG1 monoclonal antibodies is a common fragmentation pattern widely studied by size exclusion chromatography (SEC). Capillary electrophoresis with sodium dodecyl sulfate (CE-SDS) is a well-established technique commonly used for monitoring antibody fragments as well, but its comparability to SEC in monitoring hinge fragments has not been established until now. We report a characterization strategy that establishes the correlation between hinge region fragments analyzed by SEC and CE-SDS. Monoclonal antibodies with elevated hinge fragments were generated under low pH stress conditions and analyzed by SEC and CE-SDS. The masses of the fragments generated were determined by LC-MS. Electrophoretic migration of the hinge fragmentation products in CE-SDS were determined based on their mass values. Comparative assessment of fragments by SEC, and CE-SDS showed similar correlation with incubation time. This study demonstrates that CE-SDS can be employed as a surrogate technique to SEC for monitoring hinge region fragments. Most importantly, combination of these techniques can be used to obtain comprehensive understanding of fragment related characteristics of therapeutic protein products.

1. Introduction

Monoclonal antibodies have become mainstream therapeutic proteins in biotechnology. They are often expressed as tetrmeric consisting of two heavy (HC) and two light (LC) chains linked by disulfide bridges. During manufacturing and storage, cleavage of either HC or LC primary structure can occur due to different fragmentation mechanisms [1]. Cleavage of the hinge polypeptide connecting the Fab to the rest of the antibody is a well-known fragmentation pattern that occurs via hydrolysis or β-elimination as a function of the amino acid sequence, pH, temperature, and formulation buffer [2–6]. Hinge region fragmentation results in degradation of antibodies to form fragment antigen binding (Fab), fragment crystallizable (Fc), and Fc-Fab fragments, which may have negative implications for the safety and efficacy of the product [1].

Several methods can be used to monitor antibody fragments including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [7], SEC [8,9], CE-SDS [8,10], reversed-phase chromatography [11,12], and capillary electrophoresis with mass spectrometry (CE-MS) [13,14], but hinge region fragmentation is frequently monitored by SEC [15–17]. While SEC is widely employed for monitoring antibody purity during stability and release testing, its use for monitoring hinge region fragmentation is not common.

SEC separates proteins according to their hydrodynamic radius on a stationary phase column consisting of spherical porous particles with a carefully controlled pore size using an aqueous mobile phase [18]. Optimal resolution between antibody size variants is only achieved when the variants differ in size from the antibody by about a factor of 2 [19]. In the case of antibody hinge fragmentation the resulting Fc-Fab species is ~100 kDa with a ~47 kDa Fab species. Therefore the resolution between antibody monomer (~147 kDa) and Fc-Fab are expected to be suboptimal. Furthermore, since SEC is a column based separation, non-ideal interaction between mAbs and the charged [4] or hydrophobic [20] surface sites of the sta.
tionary phase often affect the resolution, especially with antibody derived compounds such as antibody-drug conjugates (ADCs).

CE-SDS employs SDS denaturation to dissociate protein components linked by noncovalent interactions rendering non-covalently associated species separable. In CE-SDS, similar to SDS-PAGE, protein migration is driven by the surface charge induced by SDS binding, which is proportional to the protein’s molecular weight. In a sieving gel matrix, a size or hydrodynamic radius based electrophoretic separation is achieved since all the SDS-protein complexes have similar mass-to-charge ratio [21,22]. CE-SDS has been used to separate a wide range of mAb fragments from the monomer with outstanding resolution [10,23]. CE-SDS under non-reducing condition (nrCE-SDS) is used to monitor the purity of denatured intact antibodies while CE-SDS of reduced antibodies (rCE-SDS) is used to monitor intact LC, HC, and non-glycosylated HC [10].

Both nrCE-SDS and rCE-SDS can be used for monitoring antibody fragments including hinge region fragments but their correlation to SEC is still not fully understood. Ouellette et al. used nrCE-SDS to quantitate Fc-Fab species generated by copper-histidine induced hinge fragmentation but no correlation to SEC results was reported [17]. Establishing the relationship between SEC and CE-SDS in quantifying and monitoring antibody hinge fragments would allow for use of CE-SDS when SEC resolution between monomer and Fc-Fab is inadequate to consistently monitor the hinge fragment. In the present work, we determined the identity and migration pattern of hinge fragments in nrCE-SDS and rCE-SDS using stressed antibodies with elevated fragment species generated by low pH induced hinge fragmentation [15].

The pH-stressed antibodies were fractionated by SEC to enrich the target fragment peaks. Assignment of hinge fragment peaks in CE-SDS separations was achieved by co-mixing the enriched SEC fractions with the unfractionated sample, and correlating the migration time of the fragment species with their masses. Both non-reduced CE-SDS and reduced CE-SDS demonstrates strong correlation with SEC. The results presented herein is to establish confidence in using CE-SDS as an orthogonal method for monitoring antibody fragments.

2. Material and methods

Unless stated, all reagents were purchased from Sigma-Aldrich and used without further purification. Recombinant IgG1 mAbs (IgG1A and IgG1B) were expressed in Chinese Hamster Ovary (CHO) cells and purified according to established practices [24]. SEC columns were obtained from Tosoh Biosciences (King of Prussia, PA). CE capillaries were obtained from AB Sciex (Redwood City, CA). Solutions were made with distilled deionized water vacuum filtered through a 0.22 µm filter.

2.1. Generation of low pH induced hinge fragments

Low pH induced hinge fragments were generated by stressing antibody in low pH incubation buffer. The pH of the incubation buffer was adjusted by titrating with 4 M citric acid to pH 3.5. The material was incubated at 37 °C in a water bath up to 14 days. Samples were taken at 0, 3, 6, 8, and 14 days for analysis.

2.2. Size exclusion chromatography

SEC analysis was performed using a Waters HPLC system with a Waters 2996 photodiode array detector (Waters Corporation, Milford, MA). Size homogeneity was determined via chromatographic separation using two TSK GEL G3000SWx11 columns (7.8 mm ID ×30 cm; Tosoh Bioscience LLC, Montgomeryville, PA) connected in tandem. The mobile phase was composed of (sodium perchlorate (25 mM)- sodium chloride (350 mM) (pH* 6.8))- isopropyl alcohol (92.5:7.5, v/v). Total run time was 40 min at a flow rate of 0.8 mL/min. The columns were kept at room temperature and the samples were stored in the autosampler at 4 °C. Fifty micrograms were injected and chromatographic separation was monitored at 220 nm via UV detection. SEC fractionation was performed on a similar Waters HPLC system equipped with a Waters Fraction Manager-Analytical. 1 mg of protein was injected per SEC run. Fractionated samples were pooled and buffer exchanged into the formulation buffer using Amicon Ultra-15 Centrifugal Filters 3 K MWCO (EMD Millipore, Billerica MA) and stored at −80 °C until analysis.

2.3. CE-SDS under reducing and non-Reducing conditions

CE-SDS analysis was performed under non-reducing and reducing conditions according to previously reported methods [25]. For the non-reduced CE-SDS approximately 40 µg of sample was added to 80 µL of denaturing sample buffer containing 2-mercaptoethanol (IAM) followed by incubation at 70 °C for 10 min. After incubation the sample was cooled at room temperature, and transferred into micro sample tubes. For the reduced CE-SDS, samples were first reduced with DTT under denaturing conditions and alkylated. All CE-SDS separations were performed on proteumelab PA 800 plus system (AB Sciex, Redwood City, CA) using a 360 µm OD X 50 µm ID X 30.1 cm bare fused silica capillary. The effective separation length was 20 cm. Samples were introduced into the capillary by electrokinetic injection in reverse polarity at 180 V/cm for 20 s. Separation was achieved by applying 15 kV voltage (approximately 25 µA) in reverse polarity using SCIEX proprietary SDS–MW gel separation buffer. UV detection of migrating proteins was monitored at 220 nm.

2.4. Intact and reduced mass analysis

Purified SEC fractions were buffer exchanged into 10 mM phosphate. For reduced mass analysis, samples were denatured and reduced in the presence of guanidine-HCL and dithiothreitol (DTT) prior to analysis. RP-HPLC separation was performed using an injection of approximately 30 µg on a Waters BEH Phenyl 1.7 µm particle, 0.21 mm X 150 mm column (Waters Corporation, Milford, MA) and the antibody subdomains were eluted with a water/trifluoroacetic acid and acetonitrile/trifluoroacetic acid (TFA) gradient. MS analysis was carried out on an Agilent 6510 QTOF mass spectrometer (Agilent, Santa Clara CA) in positive ion mode and the raw data were converted to zero charge mass spectra using a maximum entropy deconvolution algorithm embedded in Agilent MassHunter software, version B.06.00.

3. Results and discussion

The characterization results described herein are based on hinge fragmentation in IgG1 antibodies. The term “fragment”, as used in this paper, refers to the resulting molecular species from cleavage of intact antibodies in the hinge region. As illustrated in Fig. 1a, hinge fragmentation of an IgG1 antibody with all inter-chain di-sulfide linkages conserved generates Fc-Fab (cleavage in one arm), Fab, and Fc species (cleavage at both arms). The fragmentation species can be experimentally monitored in their original state by SEC or CE-SDS under non-reducing condition. In the presence of a reducing agent, the mAb, Fc-Fab, Fc, and Fab will convert to HC, LC, 1/2Fc, and Fd (the portion of the HC included in the Fab) species (Fig. 1b), which can be monitored by CE-SDS or denaturing SEC. The objective of this study is to determine the comparability of SEC and CE-SDS techniques in monitoring Fc-Fab, Fab, Fc, 1/2Fc, and Fd fragments. The experiments were performed with two IgG1 antibodies, IgG1A and IgG1B.
The IgGs have identical primary sequence in the Fc domain but different sequence in the Fab region. These were used to establish consistency in cleavage mechanism and separation of fragments from different IgG1 molecules.

3.1. Purity profile of unstressed IgG1 A and IgG1 B by SEC, rCESDS, and nrCESDS

The SEC chromatogram of unstressed IgG1A and IgG1B (Fig. S1A, Supporting Information) showed minor amounts of fragmentation species labeled as low molecular weight, LMW 1 and LMW 2. For both antibodies, LMW2 peak (24.3 min retention time) is well separated from the main peak while LMW1 peak (21 min retention time) is less resolved. With less resolved LMW1, quantitation is subjective and less precise due to the overlapping tailing from the main peak.

The nrCESDS electropherogram of unstressed IgG1A and IgG1B shows the intact antibody and species smaller than the intact antibody (LMW) distinctly separated, Fig. S1B, Supporting information. It should be noted that the LMW species in the nrCESDS profiles are mainly native antibody subunits (i.e. light chain, heavy-light, heavy-heavy, and heavy-heavy-light) held together by non-covalent interaction in SEC but dissociated under denaturing conditions in nrCESDS. Light chain (LC), heavy chain (HC), heavy-light (HL), heavy–heavy (HH), and heavy-heavy-light (HHL) species were identified based on their relative migration. These native antibody subunits have no direct correlation with SEC LMW1 and LMW2 species as they are not hinge cleavage related. The resolution of various antibody subunits suggests that Fc-Fab, Fab, Fc, and Fd fragments should separate from the intact antibody in nrCESDS.

The rCESDS electropherograms of reduced IgG1A and IgG1B comprises of LC, HC, and heavy chain without glycosylation (NGH), Fig. S1C, Supporting Information. There are minor species between LC and NGH, and post HC. The species intermediate between LC and NGH are known to occur from clipping of antibody primary structure [1,17] while the post HC peaks are non-reducible species likely composed of thioether linked antibody subunits [26] or more rarely, additional nonspecific glycosylation on HC [27].

3.2. Qualitative comparison of IgG1 hinge fragments analyzed by SEC and CE-SDS

Characterization of the fragment peaks observed in SEC and CE-SDS (with and without reduction) was performed with antibodies stressed under acidic pH 3.5 at 37 °C for 14 days. The acidic pH stress condition is known to generate increased level of hinge fragmentation [15]. The stressed samples were collected at different time points between 0 and 14 days to evaluate temporal change in fragmentation. Day 0 corresponds to time = 0 for the stress experiment. In general, similar characteristics with respect to the hinge cleavage site, SEC profile, and CE-SDS profiles were observed for both IgG1A and IgG1 B. Most of the results shown are those from IgG1A. Additional IgG1B results, including SEC and CE-SDS profiles of stressed...
IgG1B (Fig. S2, Supporting Information) are reported as supporting information.

### 3.2.1 Confirmation of SEC low molecular weight fragments by mass spectrometry

Fig. 2 shows the SEC chromatogram of stressed IgG1A from Day 0 to 14 with an apparent increase in LMW1 and LMW2 over time. A concurrent increase in aggregate peaks, HMW1 and HMW2, was also observed. The absolute levels of observed species are reported in Table S1, Supporting Information. To confirm the identity of LMW1 and LMW2 as true products of hinge region fragmentation, the Day-14 sample was fractionated by SEC to isolate the fragment peaks (Fig. S3, Supporting Information). The fractions were analyzed by reverse phase liquid chromatography coupled to mass spectrometry. Table 1 shows the most abundant species observed in the Main, LMW1, and LMW2 fractions with the expected and observed mass values before and after reduction. The intact mass analysis showed that the SEC Main fraction is predominantly intact antibody with very low level subunits that are non-covalently associated. Accurate mass determination for the minor species was difficult due to low MS sensitivity. However, there was evidence of HHL, HH, and L subunits present at very low level in the SEC Main fraction according to the nrCE-SDS profile (Fig. S4, Supporting Information). This observation is expected since SEC is run under non-denaturing conditions and antibody subunits assembled via strong non-covalent interactions will elute in the main peak.

Non-reduced mass analysis of LMW1 fraction showed a dominant mass value of 100,716 Da, which correlates with the expected mass for Fc-Fab species. The most abundant mass value in LMW2 fraction is 46,971 Da, which is in agreement with the expected mass of Fab. The detected level of the mass corresponding to Fc species in LMW2 is negligible; indicating little to no occurrence of double HC fragmentation. Upon reduction, the SEC main fraction was mainly composed of HC and LC, while the reduced mass values for LMW1 and LMW2 indicate cleavage occurring in the hinge region. The observed dominant mass species of 26,767 Da (1/2Fc) and 23,464 Da (Fd) from LMW1 and LMW2, respectively, are consistent with the expected masses. No light chain cleavage was detected. The mass analysis results showed similar low pH induced hinge fragments for IgG1B (Table S2, Supporting Information) despite differences in the Fab primary sequence.

### 3.2.2 Fragments by nrCE-SDS

The time dependent stressed samples of IgG1A was also analyzed by nrCE-SDS. The results showed apparent increase in the HC and HH peak regions over time, Fig. 3a. A slight increase, 0.8%, was also observed in the HHL peak region, which may be due to non-specific cleavage below the hinge interchain di-sulfide or above the Fab interchain di-sulfide resulting in fragments similar to HHL in size. The increase in HC and HH regions is caused by hinge fragmentation species co-migrating with native HC and HH subunits. Confirmatory experiments were conducted by spiking isolated LMW1 and LMW2 fractions into unstressed nominal samples of IgG1A followed by nrCE-SDS analysis. The nrCE-SDS electropherograms of the spiked samples are shown in Fig. 3b. The samples spiked with LMW1 showed increase in the HH peak region, which suggests that LMW1 molecular composition is similar to HH in size. The sample spiked with LMW2 showed increase in the region corresponding to HC peak, indicating that LMW2 is about the same size as HC. This observation is consistent with the SEC and MS results, and thus confirmed that Fc-Fab migrates in the HH region while Fab migrates in the HC region. nrCE-SDS electrophorograms of pure SEC LMW1 and LMW2 fractions are shown in Fig. S5, Supporting Information.

### 3.2.3 Fragments by rCE-SDS

Fig. 4a shows the electropherogram of the stressed samples of IgG1A after reduction and analysis by rCE-SDS. The result shows the fragment peaks in Regions I and II of the electrophorograms increasing with time while Region III, which probably consists of non-reducible species held together by thiether bond [26].
Table 1
Observed mass values of the dominant species in SEC LMW1 and LMW2 fractions of low pH stressed IgG1 A analyzed by LC–MS. (Mass values for IgG1 B fractions are reported in Table S2, Supporting Information).

<table>
<thead>
<tr>
<th>Dominant Species observed by LC–MS (mass values are within 30 ppm accuracy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Reduction SEC Fractions</td>
</tr>
<tr>
<td>Intact mAb</td>
</tr>
<tr>
<td>(147,670 Da)</td>
</tr>
<tr>
<td>Fe-Fab</td>
</tr>
<tr>
<td>(100,716 Da)</td>
</tr>
<tr>
<td>Fab</td>
</tr>
<tr>
<td>(46,971 Da)</td>
</tr>
<tr>
<td>After Reduction SEC Fractions</td>
</tr>
<tr>
<td>HC</td>
</tr>
<tr>
<td>(50,212 Da)</td>
</tr>
<tr>
<td>LC</td>
</tr>
<tr>
<td>(23,517 Da)</td>
</tr>
<tr>
<td>1/2Fc</td>
</tr>
<tr>
<td>(26,767 Da)</td>
</tr>
<tr>
<td>Fd</td>
</tr>
<tr>
<td>(23,464 Da)</td>
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Fig. 4. rCE-SDS electropherogram of (A) IgG1A after low pH incubation from 0 – 14 days, (B) IgG1A and IgG1B spiked with isolated SEC LMW1 and LMW2. Migration of Fd species in rCESDS for IgG1A and IgG1B are different. LC: Light Chain, HC: Heavy Chain, NGH: Non-glycosylated Heavy Chain, NR: Non-reducible species.

remained unchanged. In addition to 1/2Fc and Fd, other minor fragments are present in Region I and II. The fragment heterogeneity in these regions indicates possibility of multiple non-specific cleavages. The rCE-SDS electropherograms of the Main, LMW1, and
LMW2 fractions for Day 14 stressed sample (Fig. S6, Supporting Information) indicate that the Main fraction is comprised of HC, LC and a low level of 1/2Fc. LMW1 was reduced to LC, HC, and 1/2Fc while LMW2 was reduced to LC and Fd species. This is also in agreement with the reduced mass results. Fd and 1/2Fc are HC fragments and should lead to decrease in relative HC peak area. No change is expected in the LC level.

Similar to the nrCE-SDS experiments, the peaks corresponding to 1/2Fc and Fd species were confirmed by spiking LMW1 and LMW2 fractions into a unfraccionated, unstanched sample. The spiked electropherograms, Fig. 4b top row, showed 1/2Fc fragment migrating between LC and NGH. This is consistent with expected migration as the size of 1/2Fc fragment with the accompanying glycan structure is slightly higher than LC. However, an interesting phenomenon was observed in the migration of Fd species, Fig. 4b, bottom row. Fd fragment of IgG1A migrates after LC while Fd fragment of IgG1B migrates before LC despite having similar size. The mechanism underlying this phenomenon is not yet clear. While CE-SDS is a size-based separation, protein-SDS stoichiometry or non-ideal interaction with the CE-SDS sieving matrix could impact differential migration of species of similar size. This observation suggests that Fd fragments could migrate as a shoulder peak before or after the LC. Therefore, identification of Fd based on migration time should be done with prior knowledge of the relative migration for a given antibody.

3.3. Correlation of IgG1 hinge fragments measured by SEC and CE-SDS

For hinge fragmentation, the following schematic (Chart 1) illustrates the relationship between the expected species present at any given time before and after reduction.

After reduction, the relative amount of LC remains constant at any given time since no LC degradation is expected. Quantitative comparison of CE-SDS and SEC fragment was performed with the stressed IgG1A samples collected on Days 0, 3, 6, 8 and 14, and analyzed by SEC, nrCE-SDS, and rCE-SDS. Table 2 shows the relative abundance of the total SEC LMW, total nrCE-SDS LMW, and the total rCE-SDS region I and II species. As expected, nrCE-SDS LMW levels are higher than SEC LMW level due to the contribution of the antibody subunits and hinge fragments that are noncovalently associated and elute in the SEC Main, and perhaps HMW peaks. For example, significant increase in hinge fragment species was observed in the high molecular weight (HMW) fractions of the stressed antibody (Fig. S7, Supporting Information). The small difference in nrCE-SDS and rCE-SDS values can be attributed to minor cleavage spots that remained linked by disulfide linkages in nrCE-SDS but are decoupled upon reduction in rCE-SDS.

For correlation assessment, the time series values in Table 1 were Day 0 adjusted by subtracting Day 0 value to eliminate the contribution from native antibody subunits present at time zero, especially in nrCE-SDS, and account for de novo fragmentation. The Day 0 adjusted fragment levels by nrCE-SDS and rCE-SDS correlates linearly with SEC with R² values greater than 0.99, respectively. The regression plot for Day 0 adjusted fragments by SEC and CE-SDS is reported in Fig. S8, Supporting Information. This result is of practical importance for considering nrCE-SDS or rCE-SDS as orthogonal approaches for monitoring fragment. The linear correlation between the methods, demonstrates that CE-SDS (reduced and non-reduced) can be used to monitor monomonal antibody fragments when SEC result is inadequate.

4. Conclusion

In this paper, we demonstrate the potential of nrCE-SDS or rCE-SDS for monitoring hinge fragmentation. We correlated fragments levels observed in SEC to peaks observed in CE-SDS electropherogram. Each method exhibits unique but complementary characteristics to be considered for fragment analysis. In commonly used SEC, fragments that are strongly bound to the intact antibody and high molecular weight species remained inseparable and could potentially lead to underestimation of fragment population if those were not accounted for. Additionally, non-ideal interaction with the stationary phase could impact the Fc-Fab resolution. nrCE-SDS provides good measure of the intact antibody population devoid of non-covalently linked fragments, but co-migration of Fc-Fab and Fab fragments with antibody subunits, H and HH, and HHL can interfere with the quantitative results. We, however, showed that change in the total fragment peak area relative to the reference sample can be used to monitor fragmentation in stability studies. rCE-SDS presents some advantage over SEC and nrCE-SDS in that 1/2Fc and Fd are distinctly separated from other species. Another unique feature of rCE-SDS is that non-glycosylated heavy chain can be captured. In addition, any fragment species held together by disulfide linkages becomes apparent as well. Key consideration for monitoring fragment with rCE-SDS is the loss of intact antibody information after reduction. Nevertheless, the time dependent fragment analysis demonstrates linear correlation between hinge fragmentation levels measured by CE-SDS and SEC methods. As a result, nrCE-SDS and rCE-SDS should provide adequate information on antibody purity where SEC is not applicable. We expect that the results presented herein will further spur interest in considering nrCE-SDS or rCE-SDS as a surrogate method for monitoring antibody fragments including Fc-Fab, Fab, and Fc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.jpba.2017.06.006.
References

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