analytical chemistry

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Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.8b04396 • Publication Date (Web): 19 Nov 2018

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

A Middle-Up Approach with Online Capillary Isoelectric Focusing-Mass Spectrometry for In-depth Characterization of Cetuximab Charge Heterogeneity

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ABSTRACT

Previously, we reported a new online capillary isoelectric focusing-mass spectrometry (CIEF-MS) method for intact monoclonal antibody (mAb) charge variant analysis using an electrokinetically pumped sheath-flow nanospray ion source on a time-of-flight (TOF) MS with a pressure-assisted chemical mobilization. The direct online CIEF-MS method exhibited excellent charge variants resolution conforming to those of imaged CIEF-UV (iCIEF-UV). However, for complex mAbs, CIEF-MS spectra of the intact charge variant peaks may be overly convoluted to be effectively interpreted. In the current study, we implemented a middle-up approach to enhance the capability of the CIEF-MS method for characterizing complex mAbs charge variants by reducing sample complexity. To demonstrate such a strategy, we fragmented cetuximab through IdeS enzymatic cleavage and dithiothreitol (DTT) reduction. For the first time, online CIEF-MS resolved the complex charge variants of cetuximab at subunit level, corroborating the profiles obtained by iCIEF-UV. Furthermore, high resolution TOF mass spectra with high mass accuracy were obtained for the eight charge variants separated by CIEF-MS after IdeS cleavage, and for the eleven charge variants after IdeS digestion with subsequent DTT reduction. In-depth analyses revealed the identities of all charge variants, and pinpointed the causes of charge heterogeneity, which are in accord with those reported in the literature. The main sources of charge heterogeneity of cetuximab were identified as terminal lysine on the Fc domain (up to one on each single chain Fc), glycolyl neuraminic acid residues on the Fd' domain (up to two on each Fd'), and likely several deamidation species on the Fd' domain. No charge heterogeneity contribution was found from light chain. The in-depth

characterization of complex charge variants for cetuximab demonstrates the remarkable capability of this middle-up CIEF-MS approach. This novel workflow holds great potential for detecting and elucidating charge variants to help understand protein with complex charge heterogeneity.

INTRODUCTION

Charge heterogeneity is an important quality attribute of protein therapeutics. Charge variants of protein therapeutics are commonly analyzed by CIEF, capillary zone electrophoresis (CZE), and ion exchange chromatography (IEX) methods with UV detection[1-3]. Enabling online MS detection for charge variant analysis is an effective way to characterize the underlying species of each charge variant and gain insightful information about the source of charge variants, which has been an area of great interest [4-9]. In this regard, we recently developed a novel direct online CIEF-MS technique for recombinant mAb charge variant analysis [5]. The high resolution and high sensitivity of this method were demonstrated by its application in characterizing the charge variants of several marketed mAbs at intact level. As we continue to successfully implement this technique for analyzing and understanding charge heterogeneity of therapeutic mAbs, we found that, in several cases, despite the excellent pI based resolution of our CIEF-MS method, characterizing charge variants at the intact level for complex mAbs can be very challenging depending on the size and complexity of the intact molecules. Not only is detecting a deamidation species with a unit mass shift from a ~150 kDa mAb likely exceeding the performance specifications of commonplace mass spectrometers, but also heavy glycosylation can overwhelm the resolving power of mass spectrometry with overlapping peaks in the mass spectra. In addition, with the evolving biologic therapeutic modalities and versatile new protein engineering strategies, many therapeutic biologics have complex charge variant profiles, and present real challenges for characterization at intact level.

While advances in MS instrumentation are being made constantly to strive for high resolution, an amenable and practical approach for the status quo is selectively reducing sample complexity to facilitate comprehensive CIEF-MS analysis. These include simplifying the mAb molecules by selectively removing certain modifications or decreasing size of the molecules by fragmenting mAbs to subunits [10,11].

For probing the source of charge variants, carboxypeptidase B is commonly used to act upon basic amino acids, e.g. for C-terminal lysine removal. Sialidase is often used to clean up N-glycolyl neuraminic acid [11,12]. While Peptide-N-glycosidase F (PNGase F) is the most commonly used glycosidase for releasing N-glycans, endoglycosidases EndoS and EndoS2 cleave Fc N-glycans with different glycoform selectivity [13]. These sample treatments can selectively reduce the complexity of charge heterogeneity, thus facilitating data interpretation for charge variant characterization. Profile comparison before and after treatments may be used to validate the presence or absence of charge variants associated with these specific modifications [12].

For decreasing molecular size, besides chemical reduction of disulfide bond between heavy chains (HC) and light chains (LC) of mAbs, limited proteolysis, such as papain[14], lysyl endopeptidase (Lys-C)[15], and recombinantly modified SpeB [16], have been used to digest and fragment mAb molecules. Particularly, immunoglobulin-degrading enzyme of Streptococcus pyogenes (IdeS) has gained popularity as a reliable, specific, and efficient enzyme [17,18]. IdeS cleaves IgG at a single site on heavy chains below the hinge region through a simple and robust digestion procedure, yielding one antigen-binding $F(ab')_2$ domain and two single chain (scFc) subunits. The $F(ab')_2$ fragment can

be further reduced by DTT or tris(2-carboxyethyl)phosphine (TCEP) to produce two sets of antibody subunits of LC and Fd'. The resulting analysis based on these relatively large fragments of protein are often termed as middle-down or middle-up approaches [19-21]. Being promising in enhancing chromatographic separation and MS resolution, such workflow has been widely used in HPLC/MS analysis to monitor quality attributes for characterizing multiple, specific post-translational modifications (PTMs) of mAbs including deamindation[14], oxidation[22,23], C-terminal or N-terminal variants[24,25], isomerization of aspartic acid[26], and glycosylation[27-30]. Related work was reported for charge variants of mAb fragments after limited proteolysis digestion for profile comparisons or specific PTM investigation using IEX and IEX fractionation followed by HPLC/MS analysis[15,31-34]. An IEX charge variant method for papain digested mAb was validated for charge heterogeneity at mAb subunits level [35]. A charge variant screen workflow was also reported on seven mAbs based on comparative analyses of intact IgGs versus $F(ab')_2$ and scFc from IdeS digestion using IEX with UV detection[36]. As pointed out by the authors [36], however, IEX fractionation and offline HPLC/MS analysis is much needed for such screening methods due to the difficulty to identify the acidic and basic variants observed by UV. In addition, CZE-MS has been reported using middle-up approach for charge variant characterization of mAbs[37,38]. Few CIEF analysis with middle-up approach has been reported for comprehensive charge variant characterization of mAbs [12,16,39,40]. This is partially due to the challenging that limited proteolysis and reduction treatments generate multiple components for each intact variant, resulting in complex mixture that might be difficult to resolve and subject to peak overlapping. Without MS, overlapped

CIEF-UV species cannot be detected, and the peak assignments based on UV are usually speculative and ambiguous. As a result, charge variant characterization based on UV detection does not benefit from multiple mAb fragments and their respective variants. With a MS detector, on the other hand, middle-up approaches could significantly benefit MS analysis in terms of enhancing resolution, increasing sensitivity with high ionization efficiency, and simplifying data interpretation. The realization of an effective online CIEF-MS technique[5] that combines the high-resolution capability of CIEF and the unparalleled characterization power of MS enables a novel and highly effective workflow for charge variants analysis at the subunit level for complex and highly glycosylated mAbs.

Cetuximab is a marketed mAb with complex glycoforms. It contains two sites of glycosylation on the HC: one is in the Fc domain on Asn 299 and the second is located in the $F(ab')_2$ domain on Asn 88 [41]. Its complex glycosylation leads to high level of heterogeneities, and intensive characterization efforts have been made with various approaches [9,11,13,28,42-47]. Offline capillary zone electrophoresis MS coupling has been used to analyze IdeS treated cetuximab, and six charge variants were characterized for scFc and $F(ab')_2$ fragments [38,48]. Hyphenation of pH gradient IEX with MS using volatile and low ionic strength buffers as mobile phases has been reported for the charge variants separation of intact cetuximab [9]. IEX-UV chromatogram was presented, but no detailed MS data was given[9]. We previously reported the CIEF-MS separation of cetuximab at the intact level[5]. In that study, nine charge variants were resolved using our CIEF-MS method that correlated well with iCIEF-UV results. However, the complex glycosylation profile of cetuximab prevented effective mass

spectral deconvolution for the resolved intact charge variant peaks[5].

In this study, we investigated a middle-up approach by reducing sample complexity through enzymatic cleavage and chemical reduction to achieve comprehensive characterization of cetuximab charge heterogeneity. By separating charge variants at the subunit level using CIEF and attaining mass spectra with sufficient resolution for each fragmented mAb and its variant peaks, we aim to expand the capacity of our CIEF-MS technology by the demonstration of in-depth deciphering of the complex charge variants of cetuximab.

We believe that this novel work flow offers new pathway for charge variants analysis of protein therapeutics with complex charge variant profiles including heavily glycosylated mAbs and next-generation therapeutics, such as antibody-drug conjugates (ADCs) and complex Fc-fusion proteins.

EXPERIMENTAL SECTION

Reagents. Pharmalyte 3-10 (GE Healthcare), glycerol, urea, ammonium acetate, and 1 M DTT solution in water were purchased from Sigma Aldrich (St. Louis, MO). HPLC-MS grade reagents, including water, acetic acid, formic acid, ammonium hydroxide, and acetonitrile were also obtained from Sigma Aldrich. IdeS protease was purchased from Promega Corporation (Madison, WI). Cetuximab was purchased from Komtur Pharmaceuticals (Edgewater, NJ).

Online CIEF-MS. An Agilent 6224 TOF mass spectrometer was coupled with the Agilent 7100 CE (Agilent Technologies, Santa Clara, CA) using an EMASS-II CE-MS ion source (CMP Scientific Corp., Brooklyn, NY). The TOF fragmentor voltage (FV) was set at 380/200V for IdeS digested sample and 200V for IdeS plus reduction treated sample. The skimmer voltage was set at

65V and OCT 1RF Vpp was set at 750V. The drying gas temperature was set at 350°C with a gas flow at 6 L/min. An ionization voltage was set at 2 kV using the external high voltage power supply that comes with EMASS-II ion source. The electrospray emitters (1.0 mm O.D., 0.75 mm I.D., 30 μm tip size) and neutral coating PS1 capillaries (75 cm in length, 360 μm O.D., 50 μm I.D.) were purchased from CMP. The catholyte was 0.2 N ammonium hydroxide aqueous solution, and the anolyte was 1% formic acid, both containing 15% glycerol. Sheath liquid was 20% acetic acid with 25% acetonitrile. Protein samples (~0.5 mg/mL) were prepared in 1.5% Pharmalyte 3-10 with 20% glycerol. The catholyte solution was injected under 950 mbar for 10 s, which was followed by sample injection under 950 mbar for 75 s. CIEF separation was performed with a normal polarity voltage at 250 V/cm and a 10 mbar pressure applied on the capillary inlet. Other detailed CIEF-MS conditions were described previosly.[5]

Protein sample treatment. For IdeS digestion, the cetuximab sample was digested by IdeS at 37°C for 30 minutes using the protocol recommended by the manufacture. For IdeS digestion with subsequent reduction, the sample was first digested by IdeS at 37°C for 30 minutes, then reduced by 50 mM DDT at 37°C for additional 30 minutes. Samples after digestion and reduction treatment were desalted and buffer exchanged to 10 mM ammonium acetate (pH 6.5) using Amicon Ultra-10K centrifugal filter units (EMD Millipore, Billerica, MA) prior to CIEF analyses.

Data analysis. The CIEF-MS data acquisition and analysis were performed using Agilent Mass Hunter software. MS spectra were deconvoluted using Agilent Bioconfirm software (B.07.00).

Imaged CIEF-UV. The iCIEF-UV analyses were performed on an iCE3 unit

equipped with an Alcott 720 autosampler (ProteinSimple, San Jose, CA). Experimental details were described previously.[5] In brief, fluorocarbon-coated capillary cartridges were used with 80 mM phosphoric acid as the anolyte and 100 mM sodium hydroxide as the catholyte. Sample buffer contained 0.35% methyl cellulose, 4% Pharmalyte 3-10, and 2 M urea. Focusing was conducted at 1.5 kV for 1 min, followed by 3.0 kV for 8 min.

RESULTS AND DISCUSSION

Cetuximab has significant microheterogeneity with complex glycosylation on both Fc and Fab regions. As reported in our previous work, although we achieved CIEF-MS separation of nine charge variants of intact cetuximab corroborating the charge variants profile obtained by iCIEF-UV, heavy glycosylation of cetuximab with sialic acids made the mass spectrum of each intact variant difficult to be sufficiently deconvoluted. We were not able to obtain informative MS characterization of the molecule[5]. In order to decipher the charge heterogeneity of cetuximab, we developed a novel middle-up CIEF-MS work flow by simplifying charge variants at molecular level to limit them to one set of glycosylation site per variant. In our study, cetuximab was digested with IdeS protease that cleaved the IgG mAb into three units: two scFc and one $F(ab')_2$ fragments. The resulting charge variants were then resolved by CIEF and analyzed based on their MS data. To achieve in-depth characterization, the IdeS treated sample was further reduced to generate LC and Fd' fragments from $F(ab')_2$ followed by CIEF separation and online MS analysis.

Figure 1 shows the comparison of CIEF-MS and iCIEF-UV profiles of cetuximab at intact[5] and subunits levels. Consistency between charge variant

profiles from these two techniques demonstrates that our CIEF-MS method has successfully retained the pI based separation mechanism. Nine charge variants are observed at intact level[5]. Eight charge variants are detected after IdeS digestion, and eleven charge variants are separated after IdeS plus reduction treatment. Despite the excellent separation in iCIEF-UV, the UV-based technique does not render structural information and peak assignments of fragmented variants (i.e. Fd', LC, scFc, $F(ab')_2$) are not possible.

Mass spectra of the intact and fragmented cetuximab are shown in Figure 2. As can be seen, the mass spectrum of intact cetuximab (Figure 2a), does not have sufficient resolution, due to significant overlapping of signals from complex glycosylation[5]. However, distinct mass spectra with good resolution are obtained for fragmented cetuximab. Figures 2b, 2c and 2d clearly show the reduction of MS complexity and increase in MS resolutions from the intact molecule to $F(ab')_2$ and scFc after IdeS digestion, and to LC and Fd' after IdeS digestion with subsequent reduction.

CIEF-MS charge variant characterization of cetuximab after IdeS digestion. Figures 2b and 2c show the two distinct mass envelopes at m/z of 1800-2800 and 1000-1600, corresponding to the $F(ab')_2$ and scFc fragments, respectively. By setting two fragmentor voltages on the TOF mass spectrometer optimum for different size of the molecule within the same acquisition experiment (200V for ~25kDa scFc and 380V for ~100kDa $F(ab')_2$), then extracting the electropherograms at suitable mass ranges, we can differentiate the peaks associated with $F(ab')_2$ from those associated with scFc. As shown in Figure 3, four well resolved variants (peak 2, 5, 7, and 8) are found to be from scFc, and five well separated variants (peak 1, 3, 4, 6, and 7) are observed for

$F(ab')_2$. It's worth noting that the variant peaks from the two fragments are crisscrossed in the pI based electropherograms. Hence, the common adjacent peak assignment strategy used for CIEF-UV separation could be misleading. Different from the results out of intact cetuximab analysis [5], we are now able to obtain clear mass spectrum of each variant. Although peaks 4 and 5 are barely resolved under the CIEF conditions, the high resolution of the mass spectra enables explicit peak assignments. Peak 4 with a slightly higher pI is a $F(ab')_2$ species, and peak 5 is a scFc species with a lower pI.

Figure 4 shows the deconvoluted spectra of $F(ab')_2$ and scFc. In Figure 4a for scFc, there are clearly two major glycoforms with a mass difference of 162 Da. The scFc G0F peak (peak 7) is detected at 25,234 Da which is consistent with previous literature[46]. Two basic variants (peaks 2 and 5) and one acidic variant (peak 8) are observed. The acidic variant peak 8 of scFc has a small mass difference (Δm=0.4 Da) from the main peak (peak 7) which is likely due to deamidation species because other acidified modifications would result in a much large mass shift. The basic variant peak 2 of scFc matches well with common heavy chain C-terminal lysine (+K), as the mass difference is +128 Da from the main peak for both glycoforms. An additional basic variant (peak 5) containing masses of Δm+128 Da and Δm-0.4 Da with both glycoforms is also observed. This basic variant with masses representing combined species has been reported in the literature[38,48,49]. One explanation is that this variant is a mixture with multiple modifications, possibly a mixture of deamidated lysine variant and the species containing one less deamidation site than the main peak.

In Figure 4b for F(ab')$_2$, five well-separated charge variants (peaks 1, 3, 4, 6, and 7) show a distinct mass increment of 145 Da from peak 1 to peak 7. In addition, minor glycoforms with mass difference of 162 Da are observed on all charge variants. Consistent with previous literature [38,45], the observed mass difference of 145 Da (∆m+145 Da) in the glycoforms here is resulting from the replacement of a neutral galactose with an acidic N-glycolyl neuraminic acid (NGNA) group . Based on the CIEF-MS separation, peak 1 with mass value of 101,928 Da is assigned as the zero NGNA neutral glycoform F(ab')$_2$ [38]. The peaks 3, 4, 6, and 7 are identified as the F(ab')$_2$ glycoforms with one, two, three, and four NGNA residues, respectively. They are the acidic variants with lower pIs than peak 1, and are orderly separated by the CIEF-MS method with incremental numbers of NGNA residues. The additional glycoforms with small mass shift (<5Da) are pronounced for peak 3, 4, 6, and 7, indicating the presence of possible deamidation species. For example, peak 6 contains two major glycoforms: one with three NGNA residues at ∆m(145×3) Da, the other has two NGNA residues plus a likely deamidation modification with ∆m (145×2+1) Da.

Compared with the intact CIEF-MS analysis workflow [5], the IdeS digestion approach significantly reduces the cetuximab sample complexity and yields mass spectra with far less obscurity. Compared with the middle-up approach study previously reported on the same molecule, where six charge variants were characterized by off-line CZE-MS[38], we are now able to obtain more comprehensive, explicitly pI based charge variant analysis in a fully automated fashion. For the first time, cetuximab F(ab')$_2$ acidic variant series differ by a single NGNA residue are well separated using a CIEF-MS technique. This

provides valuable information for the assessment of cetuximab quality, in that sialic acid content is one of the critical quality attributes that needs to be thoroughly addressed.

CIEF-MS charge variant characterization of cetuximab after IdeS digestion followed by reduction. To pin down and verify the location of the charge variants, we treated cetuximab with DTT reduction after IdeS digestion, in order to further reduce the size of the molecule by converting the $F(ab')_2$ fragment to LC and Fd' fragments.

As shown in Figure 5a, three major distinct mass envelops are observed. Base peak electropherograms are extracted at the mass ranges based on the mass envelopes to detect each group of charge variants (Figure 5b). By examining the deconvoluted MS of each peak, all eleven charge variant peaks in the IdeS plus reduction treated cetuximab are successfully identified. Peaks 1, 5, 8, and 10 correspond to scFc. Peaks 2, 3, 4, 6, 7, 9, and 11 all contain light chain; and Peak 2 is mainly the residual Fab' fragment. Extracting base peak electropherogram to find Fd' is not applicable because the mass envelopes of Fd' variants shift significantly due to the different number of NGNA on each Fd' charge variant.

The deconvoluted MS revealed trivial mass difference ($\Delta m \leq 0.3$ Da) for the light chain peaks (m = 23,424 Da), indicating that the cetuximab light chain does not contribute to the presence of different charge variants. The charge variants of scFc (peak 1, 5, 8 and 10) are verified by the MS in Figure 6a that are identical to those observed in Figure 4a for IdeS treated sample.

Although it is not practical to construct an extracted base peak electropherogram representing Fd' as abovementioned, shown in Figure 6b,

the analysis of deconvoluted MS for all the peaks reveals the presence of Fd' charge variants in peaks of 2, 3, 4, 6, 7, 9, and 11, each of which also contained light chain. This suggests that the Fd' fragment variants are concomitant with light chain by a noncovalent association of Fd' and LC under the native CIEF conditions [39]. In Figure 6b, either ∆m+1 Da or ∆m+145 Da is observed between adjacent peaks for the seven charge variants of Fd'. The causes of these charge variants are either deamidation or addition of NGNA residues. Based on these results, it is clear that there are up to two NGNA groups on Fd'. This observation agrees well with the results obtained from $F(ab')_2$ with IdeS digested sample, where four NGNA residues are observed corresponding to two NGNA residues on each Fd' fragment. Compared with $F(ab')_2$, the reduced molecular size of Fd' greatly enhances MS resolution and facilitates data interpretation. All seven CIEF-MS peaks of Fd' charge variants in Figure 6a are identified by the high quality MS data owning to the benefit from the middle-up approach with reduced sample complexity and the separation power of the CIEF-MS method.

Based on deconvolution results, the charge variant peak 2 is assigned mainly as residual Fab' fragment due to incomplete reduction (Figure 6c). The residual Fab peak 2 is identified as neutral Fab' glycoform with no attached NGNA. Its mass of 50,966 Da is consistent with the zero NGNA neutral glycoform $F(ab')_2$ at 101,928 Da (Figure 4b). Minor charge variants of residual Fab' are also observed in peak 3 (∆m+1Da) and peak 6 (∆m+146 Da).

CONCLUSION

CIEF-MS charge variant characterization of intact mAbs represents real analytical challenges, not only for variants with relatively small mass differences, but also for heavily glycosylated mAb molecules such as cetuximab. High level of glycosylation leads to overlapping species, thus complicating mass spectra and resulting in incomplete identification. Using cetuximab as an example, here we illustrate a middle-up CIEF-MS approach that reduces sample complexity through IdeS enzymatic cleavage and chemical reduction, which is then followed by online CIEF-MS analysis, as a viable and effective solution. The CIEF-MS method that we have developed for intact charge variant analysis demonstrated excellent separation for fragmented mAb charge variants. The combination of reduced sample complexity and powerful CIEF separation greatly benefits MS resolution and data interpretation. For the first time, direct online CIEF-MS is enabled for mAb charge variants at the subunit level, and in-depth charge variant analysis of cetuximab is achieved with a single CIEF-MS analysis. All eight charge variants following IdeS cleavage and all eleven charge variants after IdeS plus reduction treatments are identified, which pinpointed the source of the variants of cetuximab at the subunit level.

This middle-up approach is a valuable workflow for in-depth, accurate identification of charge variants for complex therapeutic mAbs and related molecules. As our method is based upon the current benchmark charge variant separation technology, iCIEF, that has been widely used for quality attributes assessments, this middle-up CIEF-MS method offering rich MS information of resolved variants has the potential to become an essential tool to expand our capabilities in analyzing and understanding charge heterogeneity for therapeutic proteins.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors would like to acknowledge James Xia from CMP Scientific Corp for providing technical support on the EMASS-II CE-MS ion source and valuable discussion on the CIEF-MS experiments, as well as review of this manuscript. We thank Daron Forman and Aaron Yamniuk at Bristol-Myers Squibb for providing the cetuximab sample. We are also grateful to Harold Weller at Bristol-Myers Squibb for his support and for review of the manuscript.

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Figures

Figure 1. CIEF separation of intact and fragmented cetuximab. CIEF-MS electropherogram: (a) extracted ion at m/z 2960-3200 of intact mAb [5], (b) extracted base peak at m/z 1500-2600 of IdeS digested mAb, (c) extracted base peak at m/z 1000-2000 of IdeS plus reduction treated mAb; iCIEF-UV electropherogram: (d) intact, (e) IdeS digested, (f) IdeS plus reduction treated.

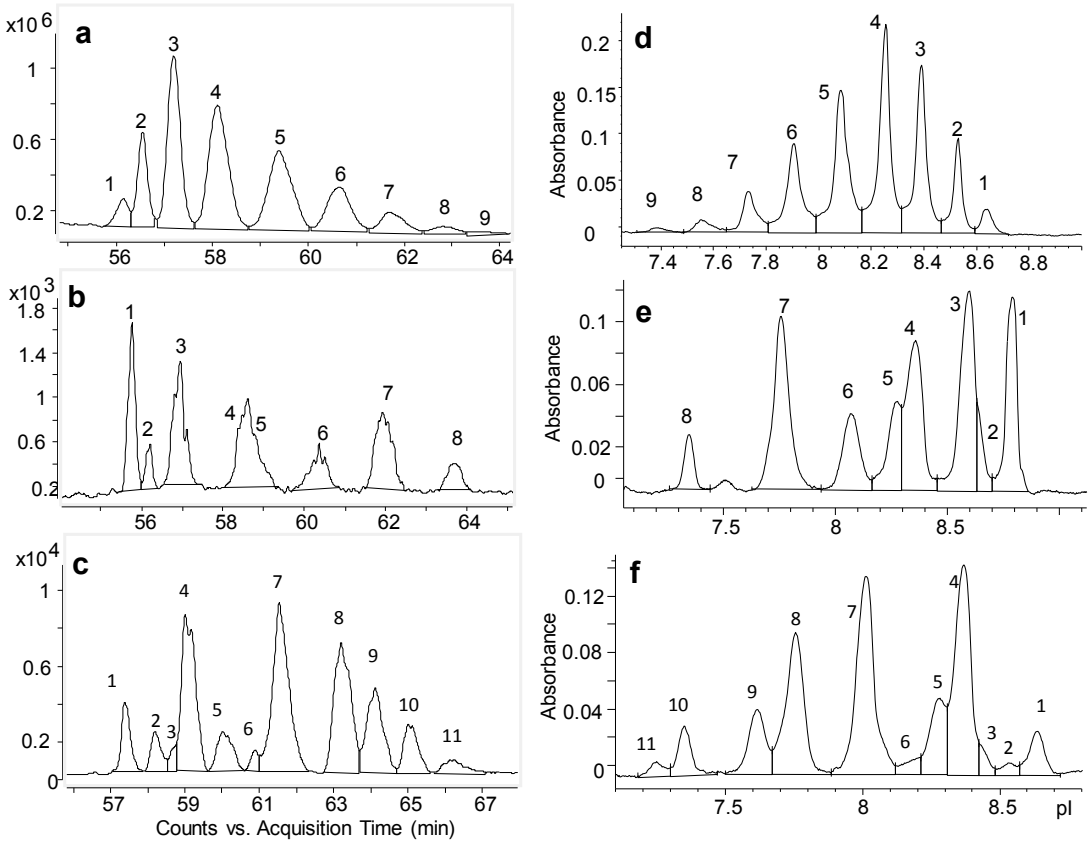


Figure 2. Mass spectra of intact and fragmented cetuximab. (a) intact, (b) $F(ab')_2$ of IdeS digested, (c) scFc of IdeS digested, (d) LC and Fd' of IdeS plus reduction treated.

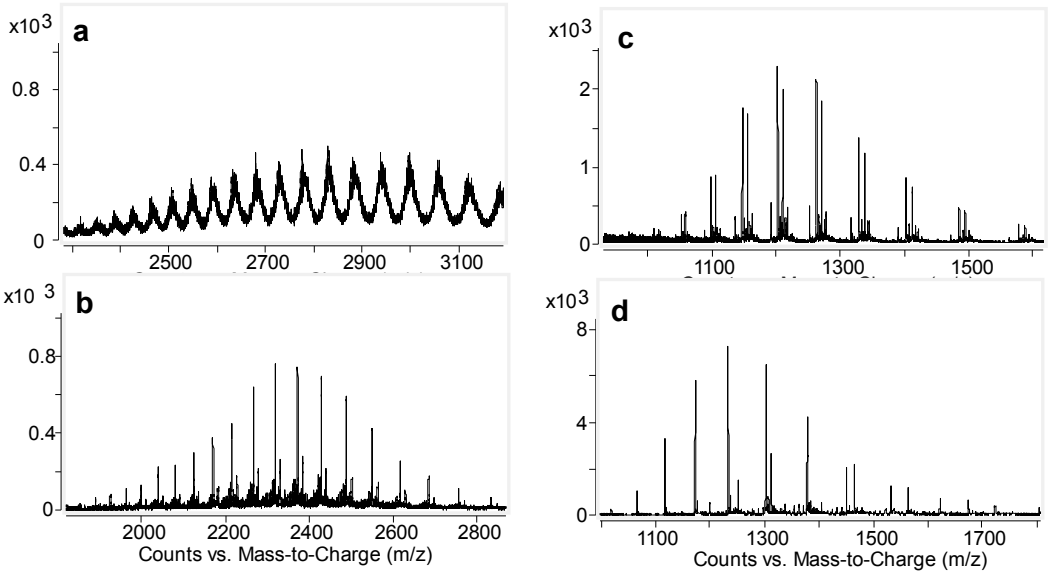


Figure 3. CIEF-MS extracted based peak electropherograms of IdeS digested cetuximab. (a) m/z 1500-2600 with FV at 380V, (b) m/z 1269-1271 with FV at 200V, (c) m/z 2486-2496 with FV at 380V.

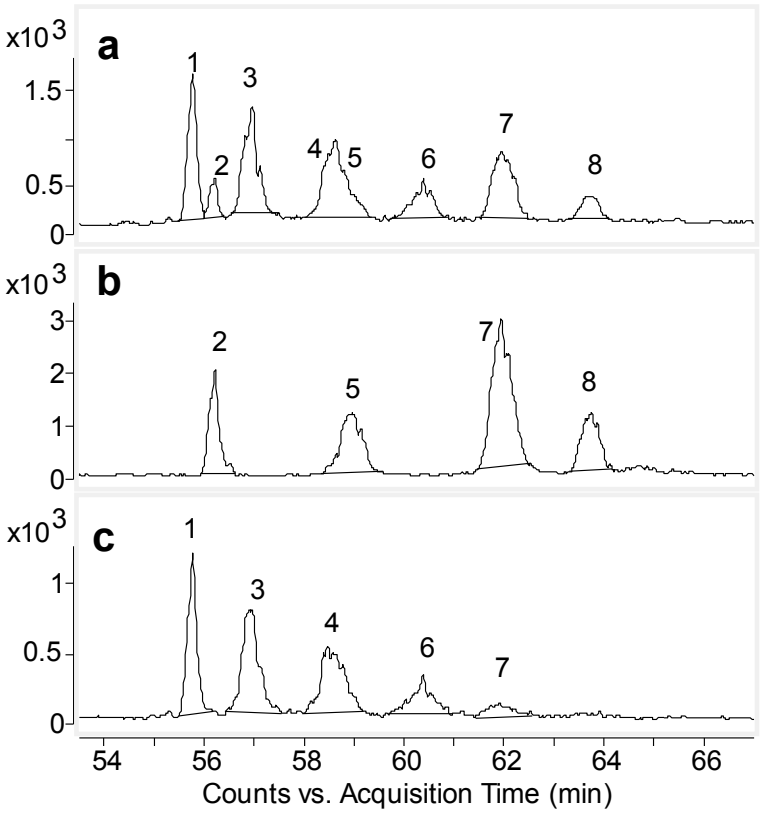


Figure 4. CIEF-MS of IdeS digested cetuximab. The deconvolued mass of charge variants of (a) scFC, and (b) $F(ab')_2$. The peak numbers correlate to the peak assignments in Figure 3.

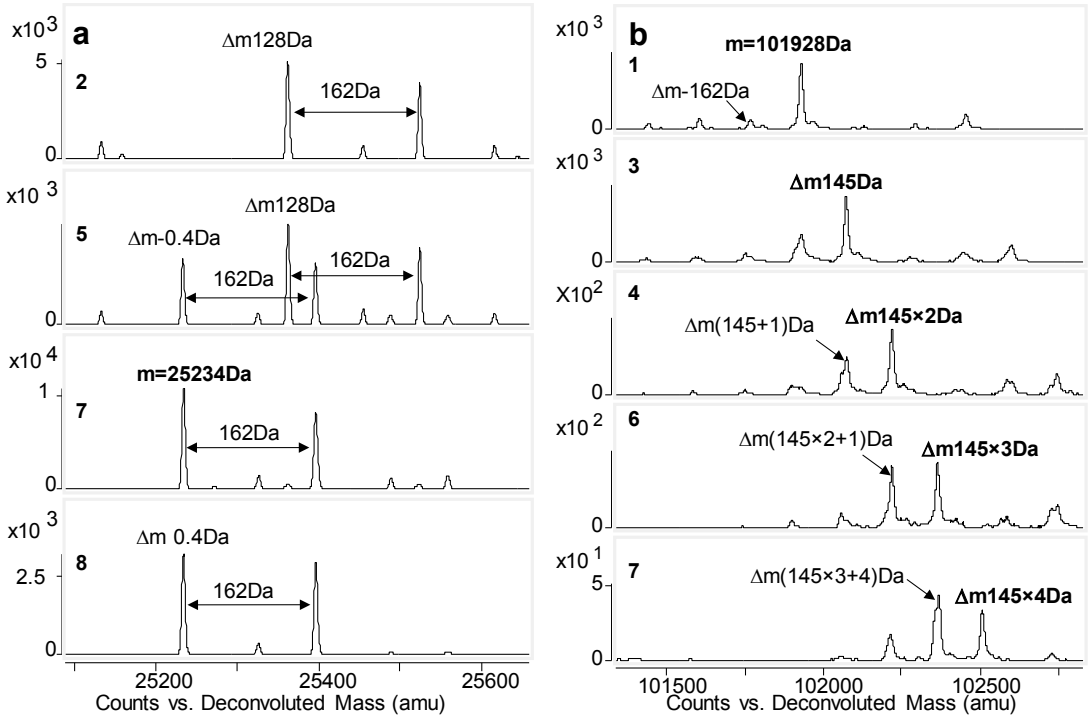


Figure 5. CIEF-MS of IdeS plus reduction treated cetuximab. (a) three major mass envelopes observed; (b) base peak electropherogram at various mass ranges, from top to bottom: m/z 1000-2000, 1234, 1209-1210, 1500.

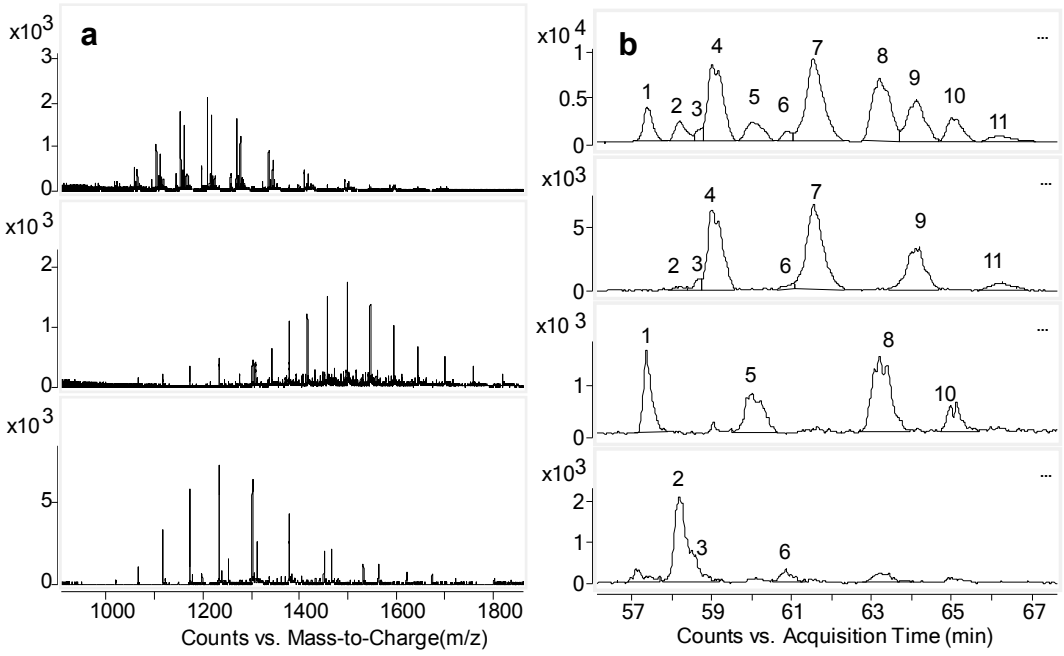
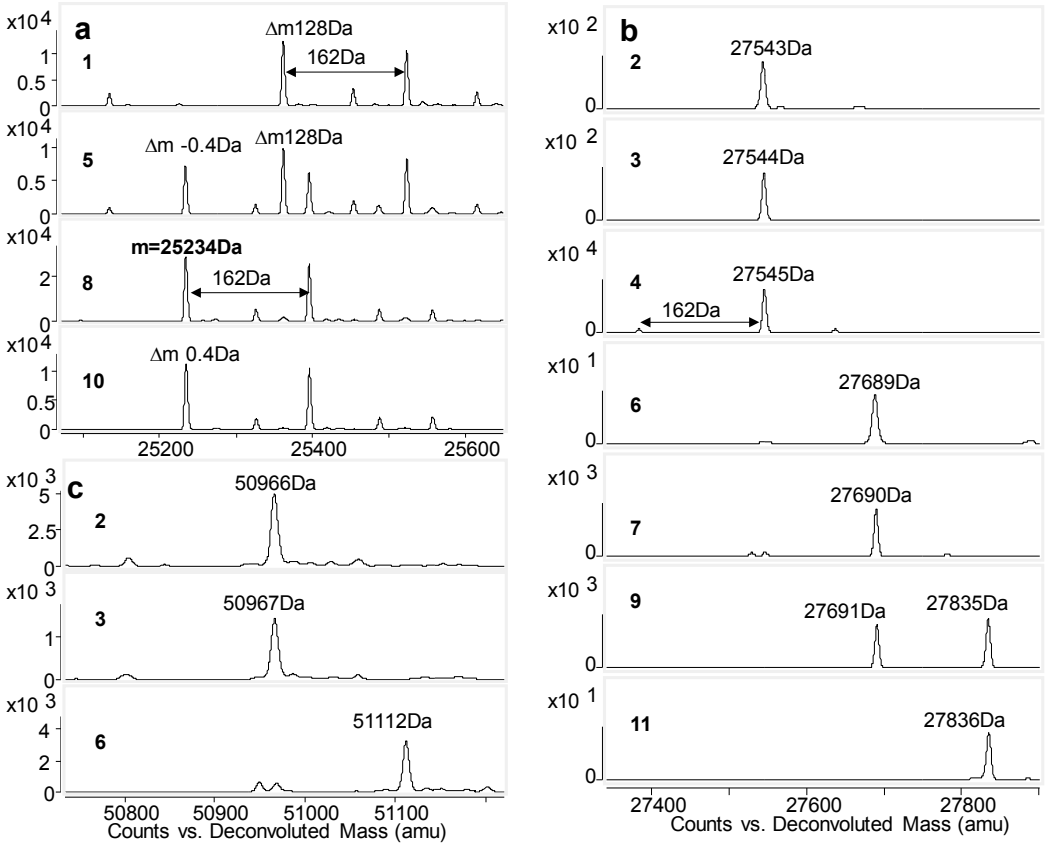


Figure 6. CIEF-MS of IdeS plus reduction treated cetuximab. The deconvolued mass of charge variants of (a) scFc; (b) Fd'; (c) residual Fab'. The peak numbers correlate to the peak assignments in Figure 5.



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