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Characterization of the acidic species of a monoclonal antibody using free flow electrophoresis fractionation and mass spectrometry

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ABSTRACT

Antibody charge heterogeneity is one of the major product-related variants in recombinant biopharmaceuticals, which has been commonly monitored by imaged capillary isoelectric focusing (icIEF). Due to the challenges with sample recovery and fractionation, other charge-based analytical approaches have been explored as complementary methods allowing for further detailed charge variant characterization. This study describes the utilization of free flow electrophoresis (FFE) fractionation in combination with other analytical techniques, such as mass spectrometry for monoclonal antibody acidic variants characterization. The preparative FFE technique allowed for continuous sample separation and fluid phase fractionation of antibody charge isoforms. The monoclonal antibody starting material was fractionated by FFE, followed by purification and characterization. icIEF analysis demonstrated the purity of the fractions and comparability of the charge profiles between these two techniques. The intact molecular mass analysis revealed that glycation modification was highly enriched in the acidic fractions. SEC UV/Fluorescence method was developed to assess the levels of aggregation and fluorescent advanced glycation end-products (AGEs). Detailed peptide map was performed and revealed that acidic fractions were enriched in AGEs, methionine, tryptophan, histidine oxidation, asparagine deamidation, lysine glycation, carboxymethyl lysine, glycine to aspartic acid substitution compared to the main peak and starting material. The results indicate that acidic variants can account for a variety of low-level modifications present as very heterogeneous forms.

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1. Introduction

Heterogeneity of recombinant monoclonal antibodies can be caused by a variety pathways, including cell culture conditions, downstream purification, formulation processing, and storage [1–3]. These product variants may negatively impact the efficacy, safety and limit the half-life. Therefore, it is critical to routinely monitor the purified antibody variants to ensure product integrity, lot to lot consistency, and stability. Charge heterogeneity analysis is one of the most important aspects in the characterization of monoclonal antibodies [4]. The charge species with lower isoelectric points (pIs) compared with that of the main species are defined as acidic variants. A number of post-translational modifications have been reported to be present in recombinant antibody acidic variants. Sialyation, deamidation of asparagine residues, succinimide formation, C-terminal lysine processing, and lysine glycation have been identified to contribute to antibody acidic variant formation [5]. Monitoring of charge variants is needed throughout the product development process to ensure product and process consistency [6]. Their detailed characterization and use in forced degradation studies have been demonstrated to assess monoclonal antibody developability [6–8]. Furthermore, characterization of charge variants to enable understanding of critical product attributes and their impact on safety and efficacy can support analytical comparability study during process changes as well as support setting phase-appropriate specifications [9,10].

There are several chromatographic and electrophoretic methods that have been developed and widely used to monitor recombinant antibody charge variants in biopharmaceutical industry, including isoelectric focusing (IEF), imaged capillary isoelectric focusing (icIEF), and ion exchange chromatography (IEX) [11–16]. Antibody charge variant separations by IEX methods apply salt concentration gradients to alter the ionic strength or utilize pH gradients with constant ionic strength. The separation mechanism is based on the surface charge differences of the antibody variants in contact with stationary phase of chromatographic columns. The IEX methods are popular for the separation of charge variants of biotherapeutics due to non-denaturing conditions and

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capability of fraction collection for further characterization. The chromatography methods can also be readily scaled up to recover and collect sufficient amount of materials for additional characterization assays. However, this approach sometimes may be limited due to variability between column lots, lack of resolution and robustness. In contrast, IEF of icIEF can resolve protein charge variants based on the pI of the molecule. The icIEF separation is conducted by migrating protein charge variants with carrier ampholytes in coated silica capillary, while different variants are focused via pH gradient by applied electric field. Compared to IEX methods, icIEF allows higher resolution, throughput and robustness, which is highly suitable for routine analysis of charge variants, and thus has become the release test assay of choice for monitoring of biopharmaceutical charge variants [15]. However, the inability to collect fractions from the capillary limits the application of this approach for further characterization by other techniques. To overcome the limitation of icIEF, multiple large-scale separation systems have been developed to allow fractionation [17–19].

In this study, we report a charge variant characterization approach utilizing free flow electrophoresis (FFE) and additional analytical techniques, such as mass spectrometry. The newly emerging preparative FFE technique allows continuous sample separation and fluid phase fractionation of antibody charge isoforms [19,20]. In FFE, the heterogeneous protein sample is continuously introduced into a separation chamber and pushed along the length of the chamber by hydrodynamic flow. As the sample travels through the separation chamber, an electric field is applied perpendicular to the hydrodynamic flow. Ampholytes present in the separation buffer create a pH gradient that leads to focusing of the charge variants based on their pI values. The focused variants are pushed out of the separation chamber and collected into 96 separate fractions. The fractions collected by FFE were further purified using protein A cartridges and the purity of each fraction was assessed by icIEF. The results showed a successful enrichment of fractions containing different acidic isoforms and the main peak. The enrichment of specific charge variants permitted the extensive identification of the acidic species by orthogonal analytical methods. A SEC UV/Fluorescence based method was developed to assess the relative the aggregation levels and abundance of fluorescent advanced glycation end products. The fractions were analyzed by intact molecular mass analysis to detect the molecular weight heterogeneity as well as to quantitate the glycation levels. Lys-C peptide map was used to characterize site-specific post translational modifications of the charge variants. The major challenge of charge variant characterization relies on the complexity of the charge heterogeneity [4,21]. A number of chemical or enzymatic modifications can occur on multiple residues in the antibody molecule, which can contribute to the charge heterogeneity. The combination of high resolution FFE fractionation technique with advanced mass spectrometry enabled detailed identification of site-specific modifications in the antibody acidic species.

2. Materials and methods

2.1. Materials

The recombinant monoclonal antibody was obtained internally at Biogen (Cambridge, MA). Mass spectrometry grade Lysyl endopeptidase was purchased from Wako Chemicals (Richmond, VA). Sequencing grade AspN endoproteinase and 1,4-dithiothreitol (DTT) were obtained from Sigma-Aldrich (St. Louis, MO). Mass spectrometry grade acetonitrile, water, and trifluoroacetic acid were purchased from Fisher Scientific (Hampton, NH).

2.2. Isoelectric focusing-free flow electrophoresis (IEF-FFE) fraction collection and purification

IEF-FFE was performed on a system from FFE service, Gmbh, Feldkirchen, Germany. The IEF separation was performed in a 100 × 500 × 0.5 mm separation chamber with 100 mM sulfuric acid and 200 mM 2-Pyridineethanol as the anodic electrode buffer, and 200 mM sodium hydroxide and 500 mM gamma-aminobutyric acid as the cathodic electrode buffer. The separation medium used to create the pH gradient for the fractionation was 1.5 % Servalyt 7–8 (Serva), 0.3 % methylcellulose (Sigma) and 3 M urea (Sigma). The separation medium was introduced into the chamber at a flow rate of 45 mL/h, while the separation chamber was cooled to 10 °C and a separation voltage of 2.9 kV was applied across the separation chamber. Samples were diluted to 1 mg/mL in the separation medium prior to fractionation and injected into the separation chamber at a rate of 1.3 mL/h. The residence times in the separation chamber were about 15 min. Fractions were collected in polypropylene 96-deep well plates. The pH-values and protein concentration for each well were measured to monitor the quality of the fractionation. The collected fractions were pH adjusted to pH 6–6.5 with acetic acid to maintain the stability of the antibody.

The FFE fractions were purified on the automated Caliper Sciclone system. Prior to purification by a protein A resin, the samples were thawed and diluted 1:4 with water to lower the viscosity of the sample and the concentration of urea. The solid phase extraction was performed with a 96-well filter plate containing 100 μL of protein A resin in each well. The resin was equilibrated with 1 mL of 75 mM phosphate, 100 mM NaCl, pH 7.4 followed by loading of the sample. The resin was then washed with 1 mL of 75 mM phosphate, 100 mM NaCl, pH 7.4 and the sample was eluted with 1.2 mL of 40 mM acetate, pH 3.5. After elution, the sample was immediately neutralized with 75 μL of 2.4 M Tris. The sample was finally concentrated with 4 mL Amicon centrifugal filters (30 kDa MWCO) from Millipore and buffer exchanged into the drug product formulation buffer (10 mM sodium citrate, 160 mM arginine−HCl, pH 6.5).

2.3. Imaged capillary isoelectric focusing (icIEF)

icIEF was performed using an iCE280 instrument and FC cartridge (5 cm x100 μm ID) from Protein Simple. For analysis by icIEF, 10 μg of the sample was diluted to 29 μL with water and added to 21 μL of a “cocktail” mixture containing 9.5 % pharmalyte pH 3–10 (GE Healthcare), 2.4 % pharmalyte pH 8–10.5 (GE Healthcare), 0.83 % methylcellulose, 2.4 % pI 5.85 marker and 2.4 % pI 8.4 marker. The sample was then thoroughly mixed, transferred into the sample vial, capped and placed in the autosampler for analysis. The anolyte and catholyte were 0.08 M H_3PO_4 and 0.1 M NaOH (both are in 0.1 % of methylcellulose solution), respectively. An electric field of 300 V/cm was applied for 1 min to prior to focusing for 8 min at field strength of 600 V/cm. The pIs of the protein isoforms were determined by calibration with the pI 5.85 and 8.4 markers, and assuming a linear pH gradient.

2.4. Analytical SEC analysis with ultraviolet-vis (UV–vis) and florescence (SEC-UV/FL) detection

The abundance of fluorescent advanced glycation end-products (AGEs) in protein sample was characterized by SEC analysis on a Waters Alliance HPLC system equipped with a variable wavelength UV detector (Waters model 2489 or 2996) and a fluorescence detector (Waters model 2475). In brief, 40 μg samples were separated on a Tosoh TSKgel G3000SWxL column (7.8 × 300 mm, 5 μm) with a in-line detection by UV at 214 nm and followed by fluorescence (FL) at excitation of 370 nm and emission of 440 nm. Mab sam-

ples were eluted within 17 min at an isocratic gradient and flow rate of 1.0 mL/min with mobile phase containing 100 mM sodium phosphate, 200 mM sodium chloride at pH 7.2.

The eluted protein peaks of monoclonal antibody samples, including the monomer peak, the aggregate peaks and the peaks of low molecular weight species, were integrated using Empower 3.0 software for both UV and fluorescence signals. The total peak areas of UV and/or fluorescence was used for the calculation of %Aggregate and %AGE, as described in results and discussion section.

2.5. Intact molecular mass analysis

The recombinant monoclonal antibody and its fractions were reduced by dithiothreitol (DTT) at a final concentration of 50 mM for 15 min. The samples were then analyzed using an ultra-high performance liquid chromatography (1290 Infinity Binary UHPLC System, Agilent) in line with an Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific). Two micrograms of each sample was injected onto a phenyl column (TSKgel Phenyl-5PW, 10 μm, 1000 Å, 2 X 75 mm, Tosoh Bioscience) equilibrated at column temperature of 55 °C. Separation of the heavy chain and light chain was achieved by a gradient of 0.03 % trifluoroacetic acid (TFA)/water (mobile phase A) and 0.024 %TFA/acetonitrile (ACN) (mobile phase B) at a flow rate of 0.4 mL/min. The separation was carried out using a step-gradient. The gradient started at 10 % mobile phase B during loading and remained at 10 % B for 1.5 min. It then ramped up to 29 % B in 0.1 min and stayed for 1.5 min. After that, it changed to 32 % in 0.1 min and stayed for 1.5 min, followed by increasing to 80 % B in 0.5 min for additional 0.5 min, before dropping to 10 % B for re-equilibration of the column for 2.5 min. Mass spectrometric settings included capillary voltage of 4 kV, capillary temperature of 350 °C, S-lens RF level 50, and an auxiliary gas heater temperature of 50 °C. The MS raw data were processed using Protein Deconvolution 3.0 (Thermo Fisher Scientific). Percentage of glycation was calculated based on the peak intensities of nonglycated and glycated species: non glycated protein (I0), protein with 1 glycation (I1), protein with 2 glycations (I2),. . ., protein with n glycations (In). The percent glycation was calculated using the following equation (weighted average): Glycation% = 100 * (I1*1+I2*2+. . .+In*n) / (I0+I1+I2+. . .+In)

2.6. Peptide map by LC–MS/MS

The recombinant monoclonal antibody and its fractions were denatured and reduced with 6 M guanidine HCl and 4 mM DTT at 25 °C for 30 min, and then diluted using 50 mM sodium phosphate, 10 mM EDTA buffer, pH 7.2. Samples were digested with endopeptidase Lys-C using a 1:10 antibody/enzyme ratio for 18−20 hours at 25 °C.

For protein digestion by Asp-N enzyme, the samples were treated with 6 M guanidine and 4 mM DTT at 25 °C for 30−60 min and then alkylated at 25 °C for 30−40 min using 20 mM iodoacetamide. Alkylation was quenched by addition of 5 mM DTT for 20−30 min at 25 °C. Digestion was performed at 37 °C for 18–20 hours with addition of Asp-N in 50 mM Tris 2 mM $CaCl_2$ pH 7.5 digestion buffer using 1:50 antibody/enzyme ratio. Following this step, second portion of Asp-N at 1:50 antibody/enzyme ratio was added to each sample for additional digestion at 37 °C for 4 h.

Digested peptides were analyzed by an ultra-performance liquid chromatography system (1200 Rapid Resolution UPLC, Agilent) coupled with a LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific). The chromatographic separation of the samples was achieved by gradient elution of mobile phase A (0.1 % TFA in water) and mobile phase B (0.1 % TFA in acetonitrile) over a HSS T3 C18 column (Waters) at the flow rate of 0.4 mL/min. The samples were loaded at 2 % mobile phase B. The gradient remained at 2 % B for 3 min and increased to 35 % B over 40 min, followed by increase to 80 % B over 2 min where it remained for 8 min. A 10-minute re-equilibration time was used between sample injections. Peptides were detected over a MS1 scan range of 400–2000 *m/z* and data-dependent MS/MS fragmentation in positive ion mode.

3. Results and discussion

Characterization of charge variants of monoclonal antibodies is challenging due to the complexity of modifications which contribute to the charge heterogeneity. This study utilized the combination of high resolution FFE fractionation technique with advanced mass spectrometry to enable a detailed identification of site-specific modifications in the antibody acidic species. The monoclonal antibody starting material was fractionated by FFE, followed by purification and characterization. The workflow of this study is shown in Fig. 1. The acidic fractions were prepared through FFE and purified using protein A cartridges. The charge variants in these fractions were characterized by a set of analytical methods. First, icIEF analysis of the fractions was performed to assess the purity and identify the enrichment of specific charge variants in each fraction. Second, SEC UV/Fluorescence method was developed to simultaneously assess the aggregation levels and fluorescent AGE. The fractions were also analyzed by intact molecular mass analysis for detection of molecular weight heterogeneity as well as quantitation of glycation levels. Finally, Lys-C peptide map was used to characterize site-specific post translational modifications of the charge variants.

3.1. Preparation of charge variant samples from free flow electrophoresis (FFE) fractions

Fractions collected by FFE were purified by protein A chromatography to remove methylcellulose, urea and the pharmalytes present in the fractions. High concentrations of urea would interfere with the protein A capture, however after a 1:4 dilution with water the concentration of urea is sufficiently low to permit efficient capture. After purification, the samples were concentrated and buffer exchanged to ensure that the samples were suitable for characterization. The recovery from the protein A capture step was high (>90 %), however the overall percent recovery is significantly lower (∼60 %) due to sample losses associated with the concentration step. The samples were first analyzed by icIEF to assess the quality of the FFE fractionation procedure and to identify the charge isoforms present in each fraction. The FFE fractions to be used for additional analysis were selected based upon their enrichment in specific charge isoforms. The FFE fractions selected for analysis of the antibody and corresponding icIEF profiles of fractions and the starting material are shown in Fig. 2. The icIEF profile for the starting material can be divided into 8 regions: 5 acidic peaks (A1-A5), 2 basic peaks (B1 and B2) and 1 main peak. Isoforms with pI differences ≥ 0.1 were well resolved and isoforms with pI differences ≥0.05 were selectively enriched. It was possible to obtain fractions highly enriched (>75 %) in A4, A5 and the main peak. The FFE fractionation was not able to separate A3 from A2 or A1 from the main peak. However, fractions containing different ratios of these acidic peaks were obtained. The relative abundance of each region in the fractions shows that the earlier fractions contained more acidic isoforms. The fractions were labeled as fraction 1–5. The most abundant isoform fraction 5 was considered the unmodified main isoform, while fractions 1 through 4 were assigned as acidic variants.

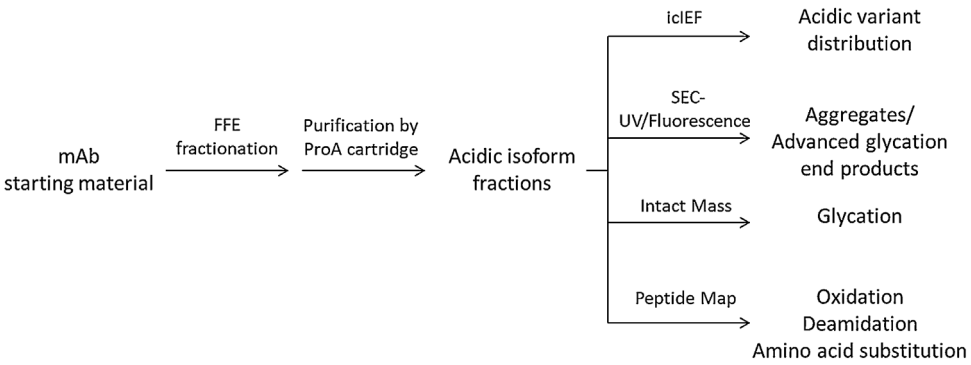


Fig. 1. Workflow of acidic variant fractionation and characterization.

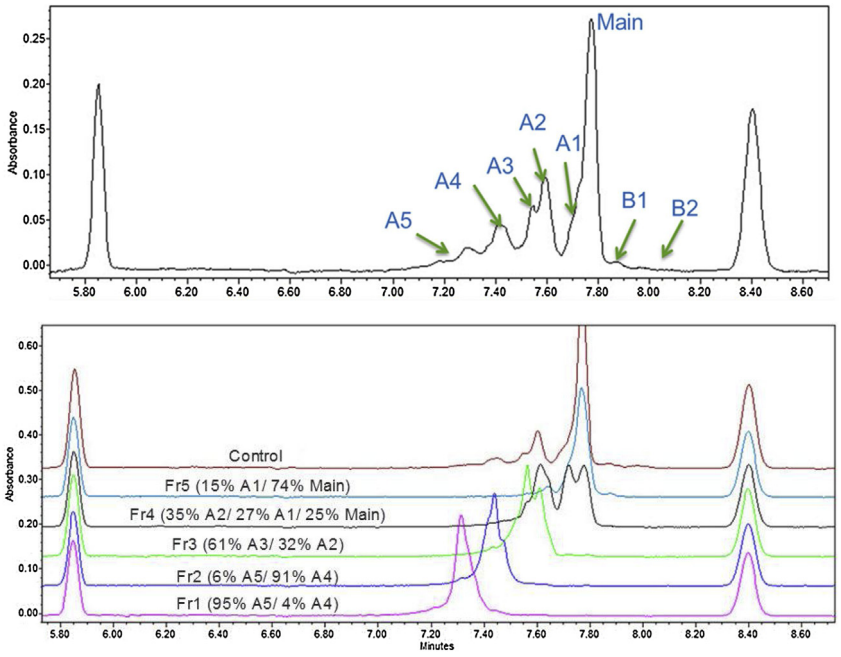


Fig. 2. icIEF analysis of fractions collected by FFE. Top panel: The five acidic variant regions identified in the mAb1 starting material icIEF profile. Bottom panel: icIEF profiles of fractions purified by FFE. The control is the starting drug substance purified material. The relative abundance of each region presents in all fractions (Fr1=Fraction 1, Fr2=fraction 2, etc) was determined by icIEF. A5= acidic fraction 5; A4=acidic fraction 4. . ..etc.

3.2. Intact molecular mass analysis by LC–MS

Glycation is a non-enzymatic post-translational modification that results in addition of a sugar to the amine group on the lysine side chain present at proteins. Therapeutic mAbs, recombinantly produced in CHO cells, are exposed to reducing sugars during cell culture and susceptible to glycation reactions [22]. Protein glycation modification occurs at the positively charge amine group of lysine side chain. The reaction between the amine group and reducing sugar forms a covalently bonded ketoamine, and results in more acidic protein species [22].

Glycation modification was detected by intact molecular mass analysis of antibody used in this study by LC–MS. The deconvoluted mass spectra showed peaks with a series of mass addition of +162 Da, which represented different degrees of glycation. The intact mass results showed the increase in glycation levels in the acidic fractions compared to main and starting material. The deconvoluted intact mass spectra are shown in Supplemental Fig. 1.

The glycation quantitation results from the analyses are summarized in Table 1. Fractions 5 (main peak) has the lowest level of glycation present at 7.2 % while the starting material contains 23.3 % glycation level. The acidic fractions 4, 3, 2 and 1 have higher levels of glycation present at approximately 35.3 %, 74.6 % 134.6 %, and 214.7 % respectively. The results demonstrate that the glycation modification is highly enriched in the acidic fractions.

3.3. SEC-UV/Fluorescence (SEC-UV/FL) analysis

Advanced glycation end-products (AGEs) are a group of complex and heterogeneous compounds, among which quite a few are fluorescent while others are non-fluorescent [23]. The cross-linked and fluorescent AGEs moieties, known to be brown color, are formed non-enzymatically by reaction of reducing sugars with the amino groups of proteins to initiate a complex series of rearrangements and dehydration with irreversible cross-linking. Fluorescent AGEs can be detected approximately at excitation around 370 nm and emission at 440 nm [24].

An SEC-UV/FL method was developed to quantify simultaneously aggregation and the abundance of fluorescent AGEs. It couples sequential detections by UV at 214 nm and fluorescence at ex370 nm/em440 nm following a size exclusion chromatography (SEC) separation [25]. The acidic fractions and the main peak fraction, as well as the starting material, were analyzed by SEC-UV/FL. The relative abundance of protein aggregation was measured by UV

Table 1
Estimate of glycation levels of the fractions by intact mass analysis.

Fractions	% Glycation [1]	% +0Gly [2]	% +1Gly [2]	% +2Gly [2]	% +3Gly [2]	% +4Gly [2]
Fraction 1	214.7	ND[3]	33.9	32.0	19.6	14.5
Fraction 2	134.6	16.4	42.4	31.4	9.8	ND[3]
Fraction 3	74.6	36.9	51.5	11.6	ND[3]	ND[3]
Fraction 4	35.3	68.9	27.0	4.1	ND[3]	ND[3]
Fraction 5	7.2	92.8	7.2	ND[3]	ND[3]	ND[3]
Starting Material	23.3	79.4	17.8	2.8	ND[3]	ND[3]

[1] Glycation percentage was a weighted average calculation based on the peak intensities of unmodified protein and glycated species only, excluding other modification and unknown species, as shown in the equation below. Among the equation, I0, I1, I2, I3 and I4 are the peak intensities of non-glycated protein, protein with 1 glycation, 2 glycations, 3 glycations and 4 glycations, respectively. %Glycation = 100 % * (I1*1+I2*2+I3*3+I4*4) / (I0+I1+I2+I3+I4).
[2] Relative composition of the glycated species in the fractions.
[3] Not detected.

Table 2
Aggregation and AGE levels from analytical SEC-AGE UV/Fluorescence of the fractions.

Fractions	% Total Aggregation	%AGEs
Fraction 1	4.4	364 %
Fraction 2	3.6	230%
Fraction 3	2.5	110 %
Fraction 4	2.4	66%
Fraction 5	2.5	22 %
Starting Material	1.3	100 %

detection in each sample. Meanwhile, the total peak area ratio of protein fluorescent FL signal in each sample was divided by the total peak area of its corresponding UV signals of the sample, and then normalized by the FL/UV ratio of the product corresponding reference standard analyzed in the same run. This normalized FL/UV result was used to determine the relative percent of total fluorescent AGEs in each sample. The quantitation results are summarized in Table 2.

The %total aggregate data in Table 2 of both acidic fractions (2.4 %–4.4 % in Fraction 1–4) and main peak fraction (2.5 % in Fraction 5) show elevated aggregation levels compared to the drug substance sample (1.3 % in Starting Material). It may indicate that the aggregates in these fractions was likely introduced during the sample fractionation and manipulation prior to the SEC analysis.

The %AGEs data in Table 2 demonstrated that the AGEs are enriched in the acidic fractions with the AGEs level at its maximum in the most acidic fraction (364 % in Fraction 1) and at its minimum in the main peak fraction (22 % in Fraction 5). The results indicate that the AGE modifications are enriched in the acidic fractions.

3.4. Peptide mapping

The fractions and starting material were analyzed by Lys-C peptide mapping to further identify the main species present in these fractions. Modifications enriched in the acidic fractions are summarized in Fig. 3. Identified modifications include advanced glycation end-products (AGEs), methionine, tryptophan, histidine oxidation, asparagine deamidation, lysine glycation, carboxymethyl lysine, and glycine to aspartic acid substitution.

3.4.1. Methionine, tryptophan, and histidine oxidation

Enrichment of oxidized species was observed in the acidic fractions including methionine, tryptophan, and histidine oxidation. The results indicate that the acidic fractions show slightly higher oxidation levels at three sites (Met95, Met253, Met429) compared to the main peak fraction. Although, the total oxidation levels may have been partly introduced during sample preparation procedures, there is a clear positive correlation of oxidation level with increase in fraction acidity.

Unlike methionine oxidation, the results indicate that tryptophan oxidation tends to be more elevated in the acidic fractions 3 and 4, respectively, which are closer to the main peak fraction.

Histidine can be also oxidized and undergo a degradation pathway to generate a number of products [26]. One of the degradants of histidine oxidation is aspartic acid. The difference of pKa of the side chain of His (6.04) and Asp (3.9) makes the modified protein to be enriched in the acidic species, which is consistent with the experimental results for the analysis of acidic fractions. The aspartic acid formation resulting from His286 oxidation at the heavy chain was indeed confirmed. The tandem mass spectrum for this modification is shown in Fig. 4.

3.4.2. Lysine modifications: glycation, Carboxymethyl Lysine (CML)

Glycation modifications present at elevated levels in acidic fractions are due to modification of basic lysine residues which alters the acidity of the protein side chain. It is known that they can further react to form advanced glycation end products (AGEs) under oxidation stressed conditions [27]. The fluorescence properties of many AGE species allows a relative quantitation of its level, as described in 3.3. section. One of the AGEs can be carboxymethyl lysine (CML) which is the non-fluorescent modification [27]. The CML modification was identified in peptide map analysis of the acidic fractions. The CML levels were enriched in the acidic fraction, whereas was undetectable in the main peak fraction. Lysine glycation levels appear to be enriched in the acidic fractions, while very low or undetectable levels were observed in the main peak fraction, which is consistent with results by SEC- UV/FL assay and intact mass assay. As described, glycation modification results in the formation of acidic species, due to neutralization of the positive charges of the lysine side chain residues.

3.4.3. Deamidation

Deamidation of asparagine (Asn) residues is one of the major modifications identified in acidic species. The amide group of Asn residues can be hydrolyzed to a free carboxylic acid (Asp), which results in the alteration of the protein charges and formation of acidic species. As shown in Fig. 3, the deamidation levels were found to be enriched in the acidic fractions at multiple asparagine sites. Asn385 and Asn390 in the heavy chain, also known as the “PENNY” peptide, are prone to be deamidated [28]. The deamidation levels are quantified as the total sum of the two residues. The deamidation levels of Asn 385 and Asn 390 show the same trend as Asn316 and are highly enriched in the acidic fractions whereas present at very low levels in the main peak fraction.

3.4.4. Amino acid substitution

Amino acid substitution in recombinant proteins is caused by DNA/mRNA mismatch during transcription, codon-anticodon mismatch during translation, or tRNA mischarging [29]. It has been reported that the most frequent base differences included a change

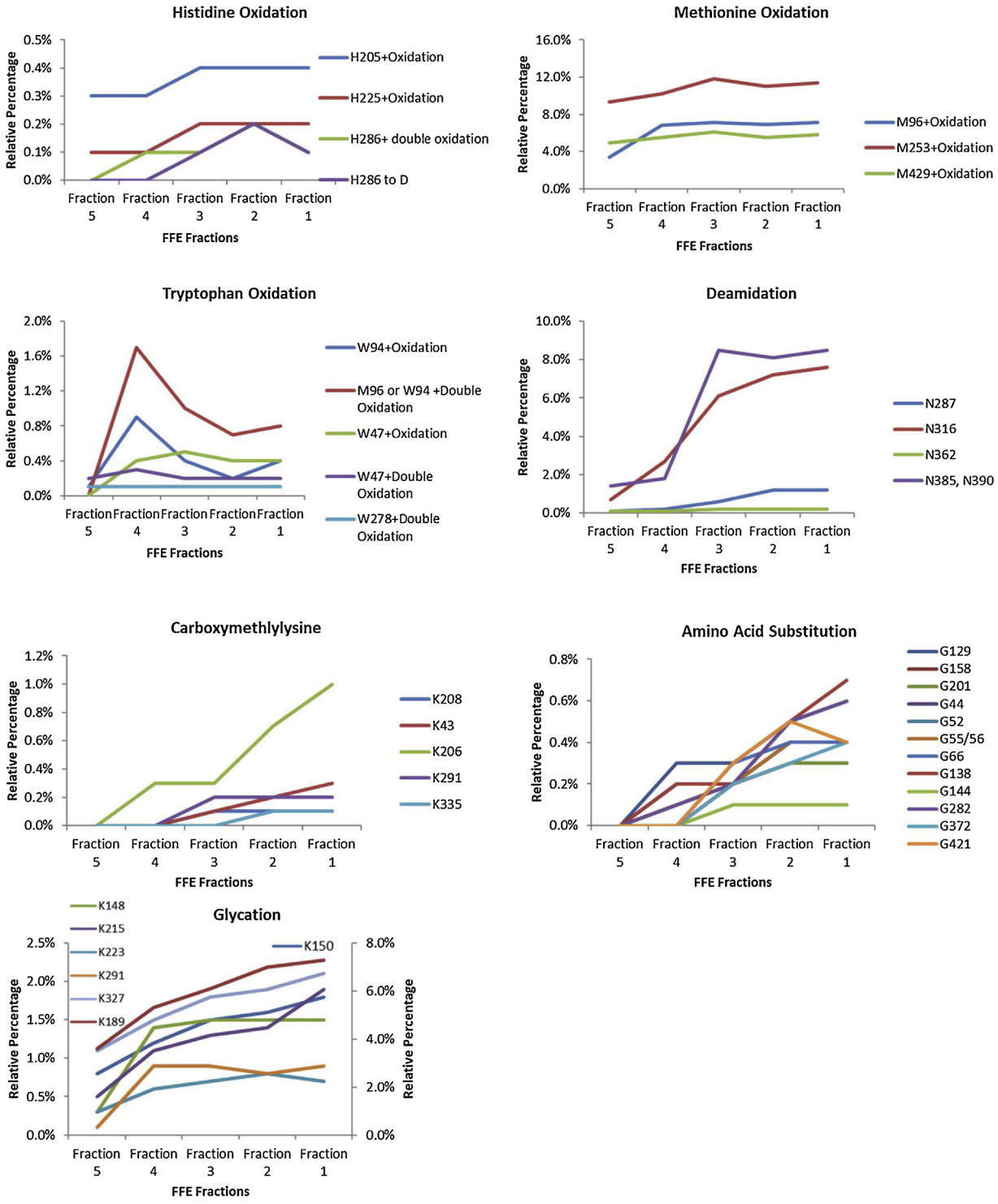


Fig. 3. Relative abundance of modification products identified in FFE fractions.

from G to A, corresponding to a G(mRNA)/U(tRNA) base pair mismatch during translation [30]. G/U mismatches during translation and transcription and C/U and/ or U/U mismatches at the wobble position during translation are key causes of amino acid misincorporation for recombinant as well natural proteins under balanced nutrient conditions [30]. In this study, Gly to Asp substitution was observed in a number of Gly residues by Lys-C peptide mapping. A tandem mass spectrum for this modification is shown in Fig. 5. This modification also results in the formation of acidic species. This amino acid substitution is measured at up to 0.7 % level in the most acidic fraction (fraction 1). The substitution shows an increasing trend among the fractions and is present at undetectable level in the main peak fraction. To further confirm the Gly to Asp substitution, Asp-N peptide mapping experiments were performed. The modified peptide due to Gly to Asp substitution should be readily cleaved by Asp-N digestion since enzyme cleaves peptide bonds N-terminal to aspartic acid residues. The peptides with Gly to Asp substitution were identified by Asp-N map results, confirming the existence of

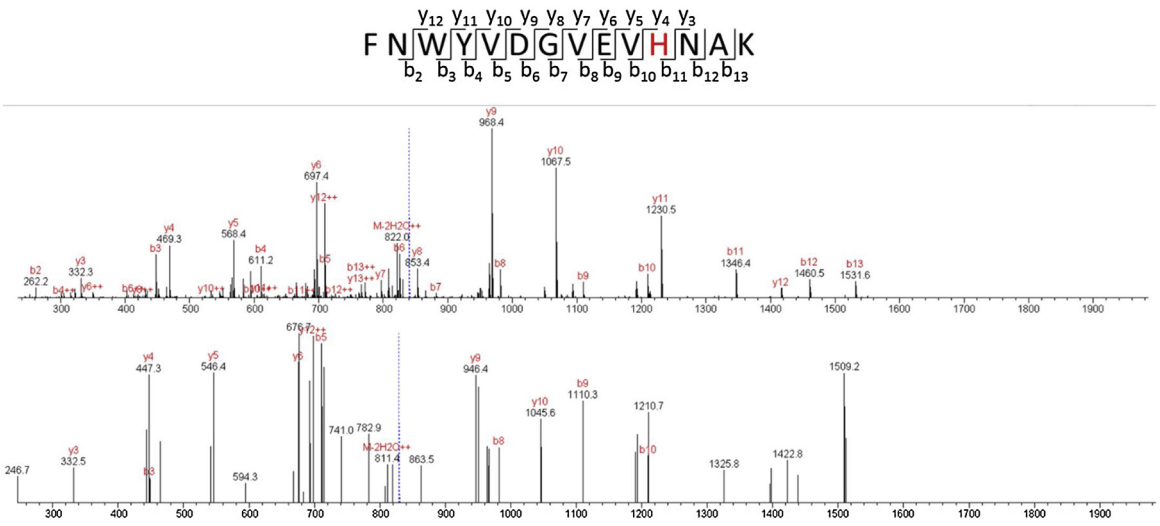


Fig. 4. Tandem mass spectra of unmodified Lys-C peptide at *m/z* 839.90 (top) with His to Asp modification at *m/z* 828.29 (bottom). The modification site highlighted in red shows the localization of His (−22 Da mass shift) to the Asp residue, which is confirmed by the b and y fragment ions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 5. Tandem mass spectra of unmodified Lys-C peptide at *m/z* 1159.07 (top) with Gly to Asp modification at *m/z* 1188.07 (bottom). The modification site highlighted in red shows the localization of Gly (+58 Da mass shift) to the Asp residue, which is confirmed by the b and y fragment ions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the modification sites whereas its levels showed that the modification was enriched in the acidic fractions (data not shown). This finding was consistent with results of Lys-C peptide mapping.

4. Conclusion

This study describes the characterization of acidic variants of monoclonal antibody by using FFE fractionation. The preparative FFE technique allows continuous sample separation and fluid phase fractionation of antibody charge isoforms. FFE can fractionate the charge isoforms based on their isoelectric point in sufficient quantities for thorough characterization by a wide range of analytical methods. icIEF analysis confirmed the identity and purity of the fractions prior to characterization. The intact molecular mass analysis revealed that glycation modification was highly enriched in the acidic fractions. The fluorescent advanced end-products were quantified by SEC-UV/FL method. Detailed peptide mapping was performed and revealed that acidic fractions are enriched in advanced glycation end-products (AGEs), methionine, tryptophan, histidine oxidation, asparagine deamidation, lysine glycation, carboxymethyl lysine, and glycine to aspartic acid substitution compared to the main peak and starting material. In conclusion, acidic variants are present as very heterogeneous forms and account for a variety of low level modifications. The workflow used in this study can be readily applied for characterization and identification of charge variants in support of biopharmaceutical process development and structure activity relationship studies.

CRediT authorship contribution statement

Suli Liu: Conceptualization, Methodology, Data curation, Writing - original draft. **Seth Madren:** Methodology, Data curation, Writing - review & editing. **Pamela Feng:** Methodology, Data curation, Writing - review & editing. **Zoran Sosic:** Supervision, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113217.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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