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Isolation and characterization of therapeutic antibody charge variants using cation exchange displacement chromatography

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ABSTRACT

In this report, we have demonstrated the isolation and enrichment of charge variants of a monoclonal antibody IgG1 using cation exchange displacement chromatography. We successfully achieved the separation of acidic, main and basic charge variants with high recovery (>70%) and purity (>90%) by using a commercially available stationary phase in conjunction with a commercially available displacer. In addition, we have isolated and enriched a trace methionine-oxidized variant of the monoclonal antibody allowing a secondary means of identification of this variant while providing sufficient enrichment for further analysis, stability tests and potency determination. Further characterization of the displacement trains by SEC indicate the possibility of enrichment of high and low molecular weight species. Glycan analysis of the displacement fractions indicates minimal variation in glycan distribution patterns among a wide spectrum of charge variants. These results provide a case study demonstrating the utility of cation exchange displacement chromatography as a viable approach to isolate and enrich antibody charge variants for enhanced molecular characterization.

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1. Introduction

The advent of quality by design (QbD) in the biotechnology industry [1] has increased the need for the isolation and enrichment of specific variants for biotherapeutic products. Critical quality attribute (CQA) assessments typically require information about safety and efficacy of specific variants or groups of variants. Isolation of these variants is essential for assessment of biophysical and biochemical properties such as *in vitro* (potency, binding) and *in vivo* (toxicity, pharmacokinetic, immunogenicity) activity. The ability to link biological function to the protein structure is critical [2].

Recombinant monoclonal antibodies (MAbs) are an important class of therapeutic proteins. MAb variants may form during any part of the antibody manufacturing process including production cell culture, downstream recovery and product formulation. Changes may also occur during manufacture and/or drug product storage, thus limiting the shelf-life of these therapeutic products. Common post-translational modifications known to occur in IgG1 therapeutic monoclonal antibodies include deamidation, methionine oxidation, N-glycosylation, glycation, pyroglutamate, c-terminal lysine, disulfide reduction and unusual linkages, fragmentation and dimerization or aggregation [3–5]. Characterization of these variants is critical because any of these changes may potentially affect the potency, immunogenicity or pharmacokinetics of the therapeutic [6]. For example, antibody charge variants may be indicative of deamidation of asparagine residues to form aspartate or isoaspartate. Those events can have an effect on potency if they occur in the binding region of the molecule, as is the case for Herceptin [3].

Most of these modifications impart a slightly different charge characteristic to the molecule. This gives rise to a charge heterogeneity that represents a spectrum of possible modifications to the primary structure of the molecule. Generally, all these modifications can alter the overall surface-charge distribution of the antibody either directly by changing the number of charged groups or indirectly by introducing structural alteration, thus modifying their ion exchange chromatographic behavior [3,5,6]. To this end, gradient ion-exchange separations are a powerful method for resolving these variants from each other. In this work, charge variants are grouped into low-p*I*, mid-p*I*, and high-p*I* isoforms. These are denoted as “acidic variants”, “main peak” and “basic variants” based on their order of elution on the analytical cation-exchange HPLC assay relative to the highest abundance main peak isoform, as shown in Fig. 1 for an IgG 1 [7]. One must keep in mind, however, that the appearance of a single peak does not necessary indicate a single variant. The discussion of isolation a “specific variant” may simply mean isolation of a specific assay “peak” which may contain one or more species as seen in the results below.

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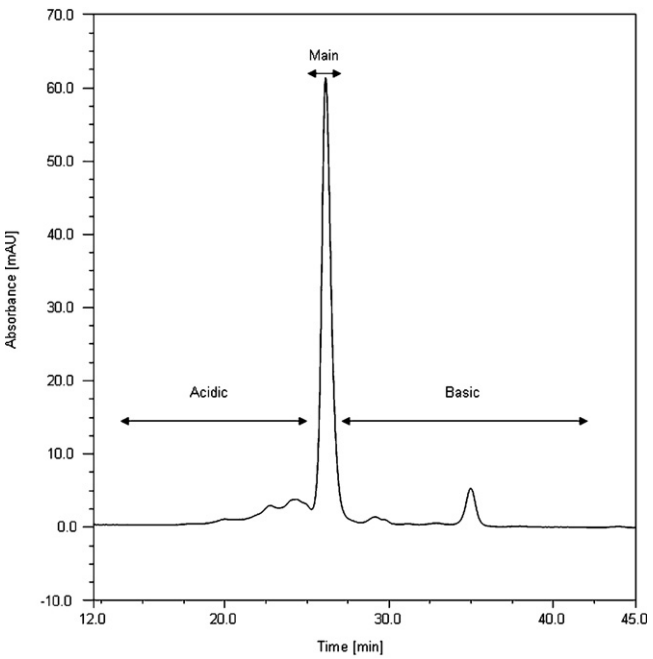

Fig. 1. Reference chromatogram of MAb on cation-exchange HPLC gradient assay. Regions of acidic variants, main peak and basic variants are identified based on order of elution.

As discussed above, isolation and analysis of specific variants is a common requirement for a robust therapeutic protein characterization package. This characterization is made difficult by the fact that some (if not most) of the charge variants exist at relatively low levels [8] and sufficient material is difficult to prepare using standard HPLC gradient chromatography techniques due to the low mass loadings required to achieve the desired selectivity.

Displacement chromatography is a technique that has been successfully utilized for the enrichment and isolation of trace variants in peptide digests [9–12], as well as high-resolution separation of proteins [13–16], oligonucleotides [17,18] and small organic molecules [19,20]. As such, displacement chromatography provides a powerful but underutilized tool in the process development and characterization of complex biological therapeutics. In an analytical capacity, displacement chromatography can provide a means of identifying trace or co-eluting species in in-process pools or final drug substance. In a preparative capacity, displacement chromatography can isolate and enrich variants for material-intensive analytical, stability and *in vivo* studies.

Displacement chromatography's powerful enrichment and selectivity provides a unique characterization tool that addresses some of the inadequacies of traditional methods of low-throughput analytical and semi-preparative methods. In this report, we demonstrate the use of cation-exchange displacement chromatography as a supplemental tool in the isolation and characterization of MAb charge variants. We also explore the selectivity of displacement towards other types of variants such as size variants and glycoforms.

2. Materials and methods

2.1. Materials

Ultra pure 2-(N-Morpholino)ethanesulfonic acid (MES) (>99.5% titration), trifluoroacetic acid (TFA), sodium chloride, potassium chloride, potassium hydroxide, sodium hydroxide, sodium tetrathionate sodium sulfite were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trypsin was obtained from Roche Diagnostics (Indianapolis, IN, USA). The IgG 1 monoclonal antibody was produced at Genentech by standard CHO cell culture process and further purified to a high purity required for pharmaceutical usage. The p*I* for the antibody was estimated by icIEF method as 8.7. Expell SP1 displacer was purchased from Sachem, Inc. (Austin, TX, USA). The 200 mm × 4 mm Mono S cation-exchange chromatography columns were obtained from GE Healthcare.

2.2. Displacement chromatographic conditions

The displacement chromatography was performed using an Agilent 1200 HPLC system with two Mono S columns (200 mm × 4 mm) operated in tandem in displacement mode to form an effective bed-height of 40 cm. The displacement equilibration buffer was 30 mM MES, pH 6.1 and 10 mM NaCl. The MAb was diluted in the equilibration buffer at 10 mg/ml and loaded at 0.2 ml/min (48 cm/h). After loading 200 mg of total MAb (40 g/L_{resin} load density), the MAb was eluted by displacement buffer at 0.2 ml/min (48 cm/h) until the protein was completely displaced. The displacement buffer was prepared from Expell SP1 displacer stock and diluted to 5 mM displacer into the equilibration buffer. After displacement, the column was regenerated by KOH and KCl wash and re-equilibrated with starting buffer. Fractions from each displacement run were collected at 3 min intervals (approximately 0.06 CV fractions) into 96 well plates. Protein concentrations were determined spectrophotometrically by absorbance at 280 nm using a NanoDrop 8000 (Thermo Scientific).

2.3. Analytical cation-exchange analysis (HPLC assay)

The cation-exchange HPLC assay used a Dionex ProPac WCX-10 analytical column (4 mm × 250 mm) packed with a nonporous pellicular resin. The chromatography was performed on an Agilent 1100 HPLC system at 34 °C with the mobile phase flow rate at 0.8 ml/min. Separation was obtained in 20 mM MES buffer and 1 mM EDTA at pH 6.0 with a gradient of sodium chloride from 70 mM to 145 mM in 60 min. To speed up the analysis of the fractions, a shorter 29-min method with similar gradient was also utilized. Separation of variants is determined from a UV trace at 280 nm. The UV trace is integrated to determine the percentage of each variant relative to total mass.

2.4. Analytical size-exclusion analysis (SEC)

A 7.8 mm × 300 mm TSKgel G3000SWxl column (Tosoh Biosciences, part No. 08541) was operated at a flow rate of 0.5 mL/min using a 200 mM potassium phosphate, 250 mM potassium chloride pH 6.2 running buffer. The column was operated at ambient temperature. Samples were diluted in running buffer and 200 μg of antibody was injected for each sample. Absorbance at 280 nm was used to monitor levels of soluble aggregates, monomer and fragments.

2.5. Glycan analysis by CE fluorescence assay

The released oligosaccharides were labeled with 9-aminopyrene-1, 4, 6 trisulfonate (APTS), a fluorescent reagent. The labeled oligosaccharides were separated by capillary electrophoresis using an eCAP N CHO coated capillary and detected by laser-induced fluorescence (LIF). The detail of this method has been described before [21].

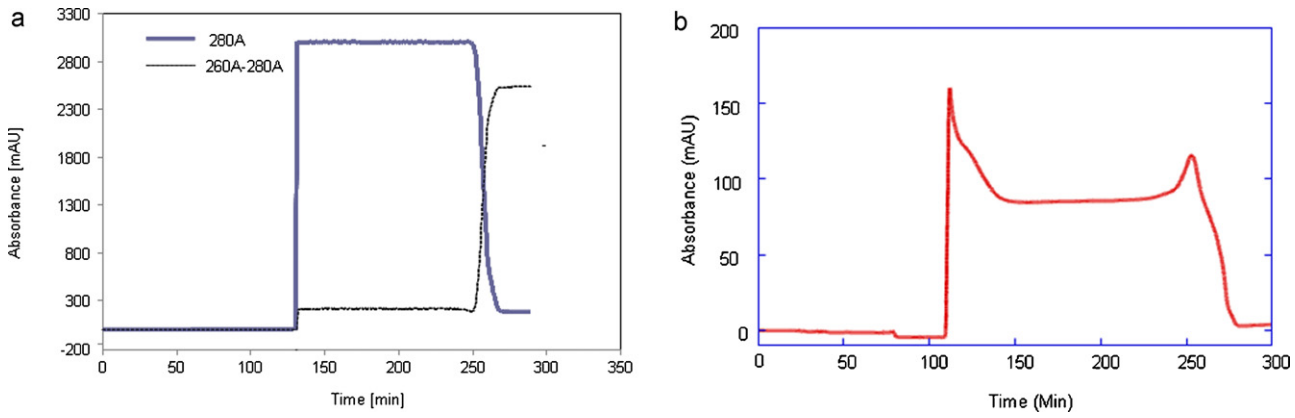

Fig. 2. (A) Representative displacement chromatogram observed at *A*280 nm (thick line) and *A*260 – *A*280 showing the displacer trace (thin line). (B) Representative displacement chromatogram at an offset absorbance (310 nm) showing evidence of transition zones in the displacement train.

2.6. Liquid chromatography electrospray time-of-flight mass spectrometry (LC–ESI-TOF) analysis of intact and reduced MAb

Liquid chromatography electrospray time-of-flight mass spectrometry (LC–ESI-TOF) was performed on an Agilent 6210 electrospray-ionization time-of-flight (ESI-TOF) mass spectrometer (Agilent, Santa Clara, CA) for analysis of intact and reduced MAb. An Agilent Nano-HPLC Chip (equivalent to Zobax 300SB-C8 analytical column of 43 mm length × 75 μm internal diameter, 5 μm particle size) was used for desalting and separation using a linear gradient from 20% B to 90% B in 6 min. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. The flow rate was at 0.4 μL/min. The injection amount was approximately 1–5 ng of protein. The effluent from the column was directed into the electrospray source of an Agilent 6210 ESI-TOF mass spectrometer. Spectra consisting of multiple charged protein ions were deconvoluted using the Agilent MassHunter Workstation Software.

2.7. LC/ESI-MS/MS analysis of trypsin digest

The trypsin digest was prepared using the sulfiltolysis procedure. 50 μl of MAb at 3 mg/ml was mixed with 85 μl of sulftolysis reagent (containing 16 mg/ml sodium sulfite and 8 mg/ml sodium tetrathionate in 6 M Guandine HCl, 360 mM Tris, 2 mM EDTA, pH 8.6). The mixture was incubated at 37 °C in a water bath for 20 min. The solution was then buffer exchanged into digestion buffer (25 mM tris, 1 mM EDTA, pH 8.3) using a desalting spin column (Pierce). Trypsin was added at a ratio of 1:25 and the mixture was incubated in a 37 °C water bath for 5 h. The digestion was quenched by adding 2.5 μl of 10% TFA. The on-line LC/ESI-MS/MS analyses were carried out using an Agilent 1100 HPLC system coupled with a Thermo Fisher (San Jose, CA, USA) LTQ mass spectrometer equipped with an electrospray ionization source. The digested antibody was injected onto a Zorbax C8 column (3.5-μm particle size, 300-Å pore size, 2.0 mm × 150 mm) at 37 °C. The peptides were separated after a 5 min hold at initial conditions with a linear gradient from 0% to 17% solvent B in 57 min, to 32% solvent B at 149 min, to 45% solvent B at 162 min, and to 95% solvent B at 173 min. Solvent A consisted of 0.1% trifluoroacetic acid (TFA) in water and solvent B consisted of 0.08% TFA in acetonitrile. The column was maintained at 35 °C and eluted at a flow rate of 0.3 mL/min. The elution profile was monitored at 214 nm and 280 nm. The mass spectrometer was operated in the data dependent mode to automatically switch between MS and MS/MS. Survey full scan MS spectra were acquired from *m*/*z* 300 to *m*/*z* 2000.

3. Results and discussion

3.1. Evaluation of displacement separation of charge variants

Due to the nature of displacement chromatography, the UV (*A*280 nm) chromatogram of a well established displacement train

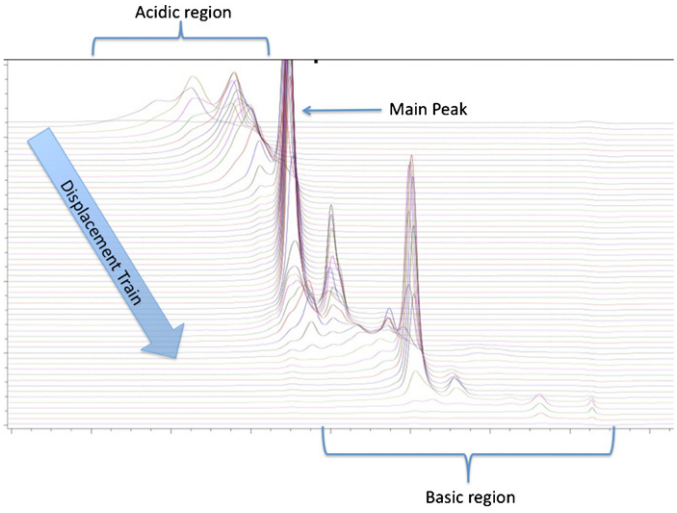

Fig. 3. Superpimosition of all analyzed displacement fractions from a single displacement run. Chromatograms are arranged back to front corresponding to the progression of the displacement train from start to finish. Fractions were analyzed using the cation-exchange HPLC gradient assay.

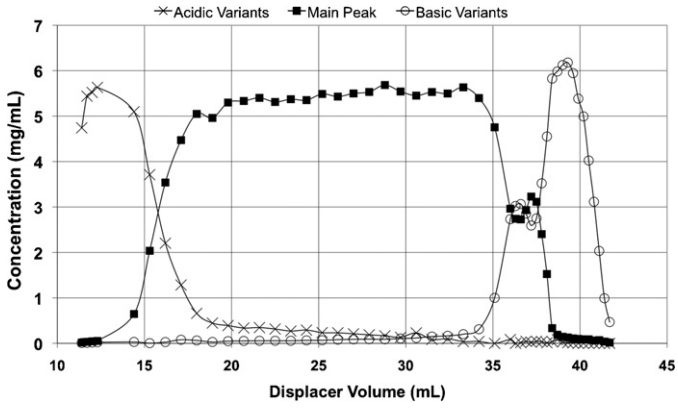

Fig. 4. Pseudo-chromatogram of cation-exchange displacement train showing MAb acidic variants, main peak and basic variant elution. Individual data point represents a fraction analyzed via the cation-exchange HPLC gradient assay.

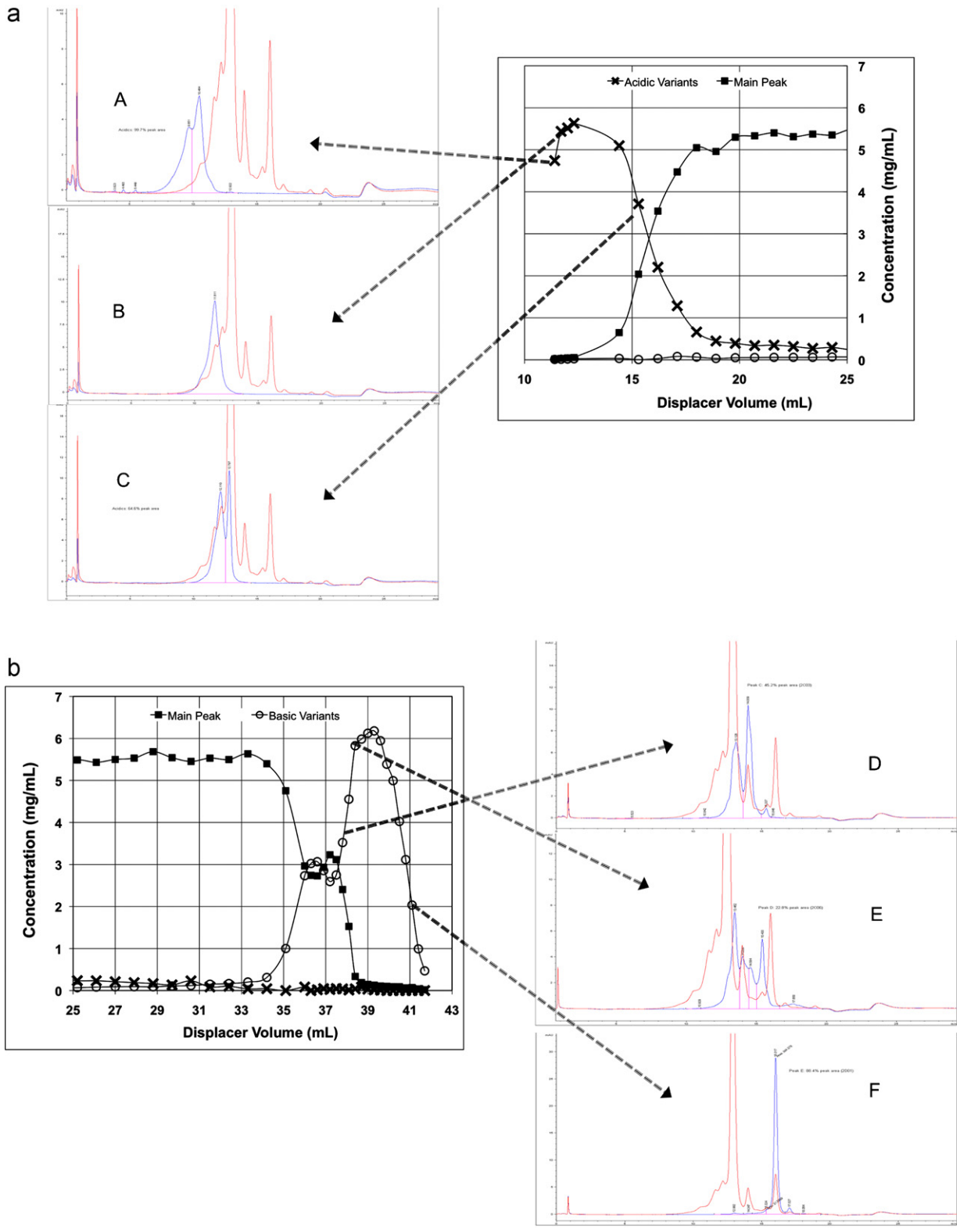

Fig. 5. (A) Acidic region of MAb displacement train showing representative fractions (a–c) analyzed via cation-exchange HPLC gradient assay. Red chromatograms (reference load material) are superimposed with blue chromatograms (displacement fraction) to indicate the progression of variants across the displacement train. (B) Basic region of MAb displacement train showing representative fractions (d–f) analyzed via cation-exchange HPLC gradient assay. Red chromatograms (reference load material) are superimposed with blue chromatograms (displacement fraction) to indicate the progression of variants across the displacement train. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

should appear to be rectangular with a step increase in concentration for each successive component that elutes from the column. However, displacement trains are highly concentrated resulting in a saturated UV detector on most standard chromatography equipment. The UV chromatogram will thus appear rectangular with no evidence of transition zones. The UV *A*280 chromatogram from the MAb displacement separation is shown in Fig. 2a. Monitoring absorbance at *A*260 can also provide the location of the

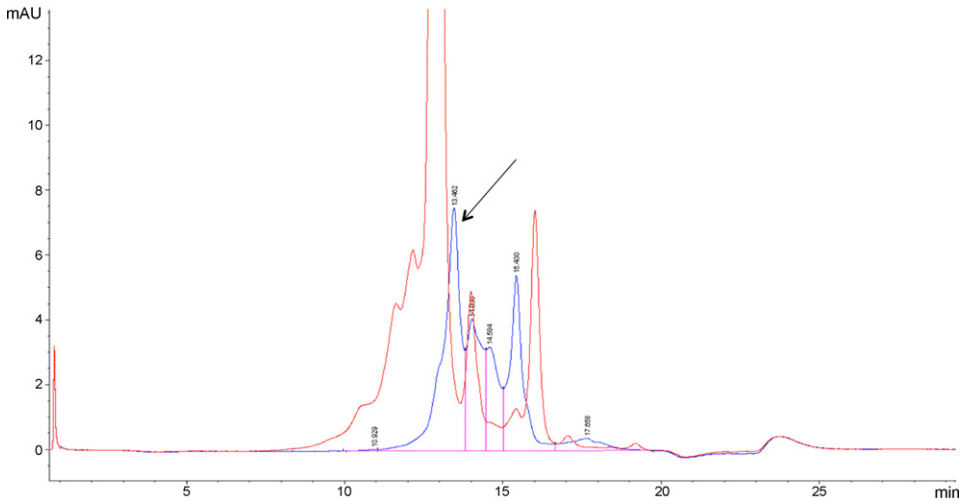

Fig. 6. Basic variant displacement fraction (from (e) in Fig. 5B) analyzed via cation-exchange HPLC gradient assay showing an enriched trace variant (blue chromatogram with arrow). Red chromatogram is the superimposed reference chromatogram (load material). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

displacer breakthrough if using the Expell SP1 Displacer. The rectangular shape of the *A*280 trace makes it difficult to detect zone transitions, making real-time pooling of the desired species difficult unless the locations of the zones are predicted *a priori*. In order to help identify the transitions of the bands, offset UV monitoring (*e.g.* away from the *A*280 maximum) may be used, as well as smaller path-length detectors. Fig. 2b shows the same displacement separation in Fig. 2a monitored at an offset absorbance (*A*310 nm) to help identify transition zones within the displacement train in real time.

While offset absorbance can help identify the general location of transition zones, identification of the actual transition zones between closely related charge variants within the displacement train required secondary analysis of the fractionated displacement train using an analytical cation-exchange HPLC method (see Section 2.3). The fractions analyzed by the HPLC assay were compared to the chromatograms of the load material to identify the variants collected in each section of the displacement train. Analytical HPLC chromatograms were overlaid to show qualitative isolation results across the displacement train. Fig. 3 shows the progression of all analyzed fractions from a single displacement train. To quantify the different species present in the displacement fractions, the peak area percentage for each charge variant (acidic, main and basic) was calculated from the area provided by the HPLC assay chromatograms via peak integration for each fraction. The variant peak area percentage in combination with the spectrophotometrically determined total protein concentration in each fraction allowed construction of a pseudo-chromatogram depicting the separation of each charge variant within the displacement train. The term pseudo-chromatogram refers to the fact that the "chromatogram" is derived from an offline analysis of fractions as opposed to an on-line detector in real-time. Fig. 4 shows a reasonably well-established displacement train. As expected for a cation exchange resin, the acidic variants (low p*I*/lower affinity) were displaced to the front of the displacement train followed by the main peak and the basic (high p*I*, highest affinity) variants. The displacement train shows overlap in the transition zones between each charge variant type most likely due to mass transfer limitations in the stationary phase and/or post column mixing in the UV detector or tubing prior to fractionation. Optimization can be performed to reduce the overlap of these zones by exploring smaller resin particles, monolith columns, slower flow rates or longer column lengths to minimize the relative overlap size with respect to the total displacement train length. Operating two 20 cm columns in tandem (to make and effective 40 cm length) significantly increased the recovery of the enriched charge variants compared to one 20 cm column (data not shown). The 20 cm column and 2 cm × 20 cm tandem column were operated at identical conditions including linear velocity and load density (40 g/L_{resin}).

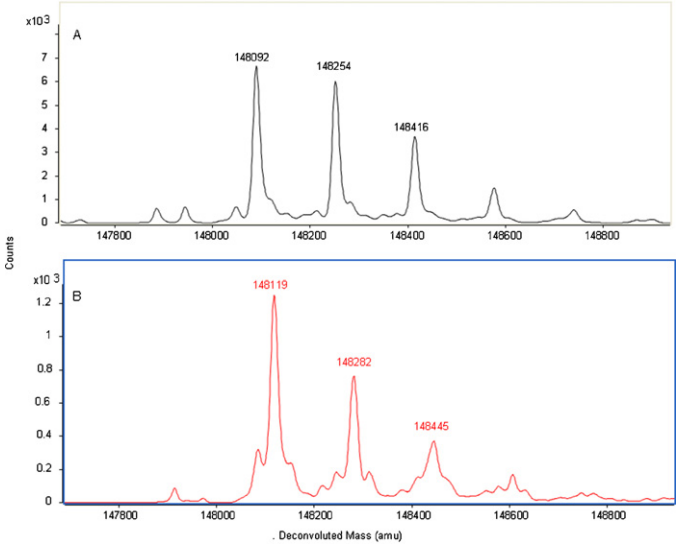

Fig. 7. On-line LC–MS analysis of the intact MAb: (a) reference MAb; (b) isolated trace variant.

Due to the overlap of species within the transition zone of the displacement train, analysis of selectivity and throughput of the separation is not straightforward. Numerical integration (using the trapezoidal method) of the pseudo-chromatogram allowed quantitative analysis of the selectivity of the separation by calculating the purity of each variant group as a function of displacement volume collected. Analysis in this manner shows the tradeoff between variant recovery and variant purity across the displacement train. This also allows one to pool the appropriate displacement fractions to create the desired purity of each variant, or to develop a pooling strategy that avoids fractionation altogether. By setting a purity target for each variant group (*e.g.* 90% pure), a recovery and throughput could be calculated for each variant at that specified purity. For this separation, 76% of total acidic variants were recovered in the displacement train at 90% purity with a throughput

Table 1
Load to displacement column was 200 mg of MAb. Purity of load material was approximately 20% acidic variants, 70% main peak and 10% basic variants.

Variant group	%Purity of pooled fractions	%Recovery from load	Mass of variant recovered (mg)	Throughput of variant (mg/h)
Acidic	90	69	21	2.8
Main	90	84	110	15
Basic	90	78	17	2.3

of 2.8 mg of acidic variants per hour. This is significantly better than our typical preparative HPLC gradient separation, which provides approximately 0.2 mg of acidic variants per hour of the same purity. A 10× increase in throughput allows faster and more efficient isolation of these MAb charge variants for various product characterization studies. Table 1 shows the mass, % recovery and throughput of each variant group at 90% purity.

Up to this point, we have lumped groups of variants as either acidic, main peak or basic variants according to the elution position of the variant on the HPLC assay. As shown in Fig. 5a and b however, there was clear isolation of individual components within the acidic variant and basic variant zones of the displacement train. This demonstrates potential to achieve fractionation of *individual* variants within the displacement train.

3.2. Isolation and identification of a trace variant

Fig. 6 reveals a charge variant that is not apparent in the reference chromatogram for this MAb. This variant showed considerable enrichment in the displacement train and provided evidence of a low-abundance charge variant in the MAb drug substance that was not detectable on the standard HPLC cation exchange assay due to its proximity of elution near the main peak.

The basic variant fraction indicated in Fig. 6 was further polished using the gradient HPLC cation-exchange assay to aid the identification of this trace specie(s). On-line LC–MS analysis of the intact MAb reference material showed multiple major molecular weights (MWs) (148,092, 148,254 and 148,416 Da) associated with different glycosylation patterns (G0, G1 and G2, respectively). The intact MWs of the enriched "new" peak were 148,119 Da (G0), 148,282 Da (G1) and 148,445 Da (G2), which were on average 32 Da more than the reference material (Fig. 7). The precision of the TOF mass spectrometer is typically less than ±5 Da for the intact antibody, which indicated this +32 Da could likely be a double-oxidation related species. This result is confirmed by mass analysis for the reduced heavy chain of the variant, which showed extra heavy chain peaks of 50,564 Da and 50,726 Da along with the normal expected peaks of 50,531 Da and 50,692 Da in comparison with the reference MAb heavy chain MWs of 50,533 Da and 50,695 Da (Fig. 8). No difference in the light chain MW was observed, indicating that the modification was mainly in the heavy chain (data not shown). Peptide mapping was employed in order to locate the exact location of the oxidation modification(s). The UV overlay of peptide mapping of the isolated variant was compared to that of the reference MAb shown in Fig. 9. As the key difference, two new peaks as P1 and P2 were visible in comparison with the reference material. The LC–MS data for the two new peptides are shown in Fig. 10 and compared with mass spectra in the same elution time for the reference MAb. For P1, the single charged ion of 851.53 Da agrees with a Met 254 oxidation in the peptide DTLMISR (AA251–AA257). For P2, the triple charged 948.29 ion is consistent with the Met 430 oxidation in peptide WQQGNVFSCSVMHEALHNHYTQK (AA419–AA441). The same ions were also observed for reference at much less intensity, indicating that these variants are present in the reference material although not detectable on the cation-exchange gradient assay. These analyses verify that both oxidations occur at the Fc sequence, which is quite common for IgG1 MAbs [6]. It is interesting to note that the methionine oxidation is not random in this variant, as indicated by the absence of a +16 Da species in the intact and reduced heavy chain. Similar phenomena have been reported for the thermo-stressed induced methionine oxidations, in which multiple methionine oxidations occur preferentially in the same heavy chain in contrast to random oxidation by tBHP treatment [6]. This points out the need for careful selection of forced degradation conditions to study methionine oxidations, as oxidation species generated by those procedures could be different from the oxidation variants in the starting reference material. Though these methionine oxidations at the Fc region might not have an impact on

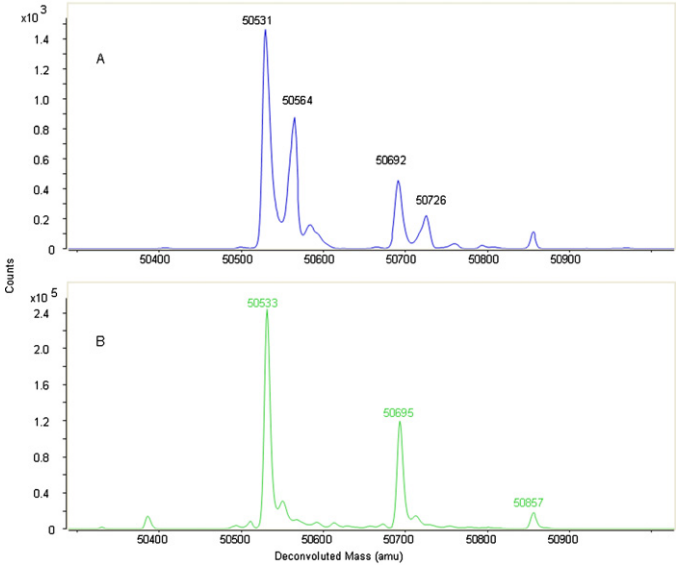

Fig. 8. On-line LC–MS analysis of the intact MAb heavy chain: (A) isolated trace variant; (B) reference MAb.

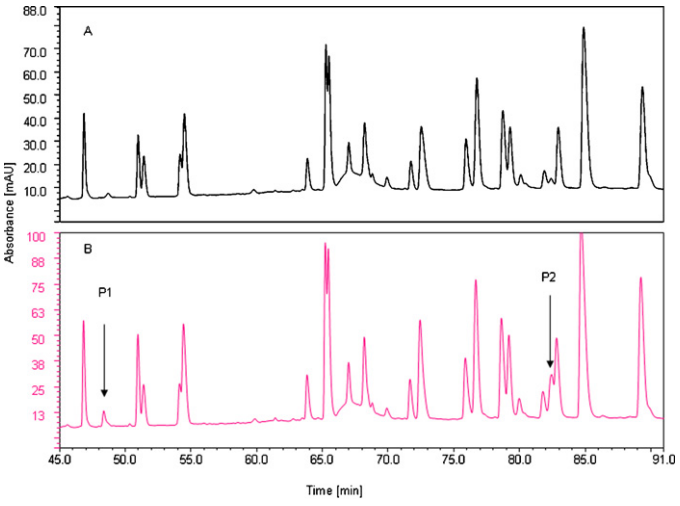

Fig. 9. Region of the peptide map of the isolated variant (A) compared to that of the reference MAb shown (B) indicating two new peptides (P1 and P2) in the isolated trace variant.

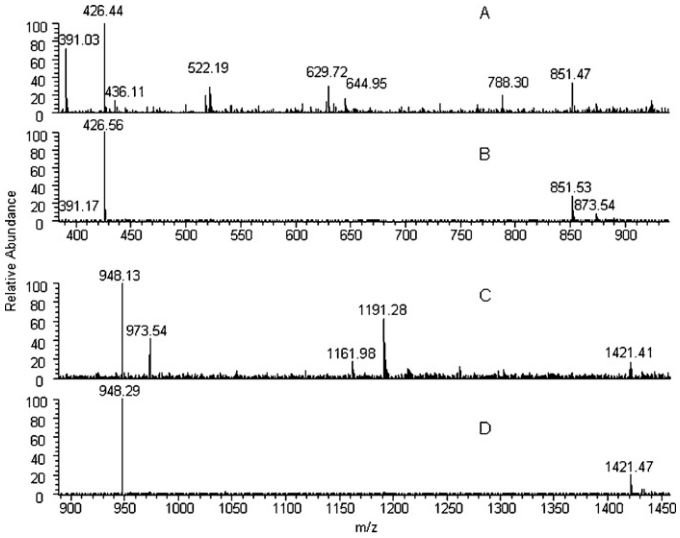

Fig. 10. LC–MS spectra for peptides "P1″ and "P2″ (B and D, respectively) compared to the reference MAb in the same LC–MS region (A and C).

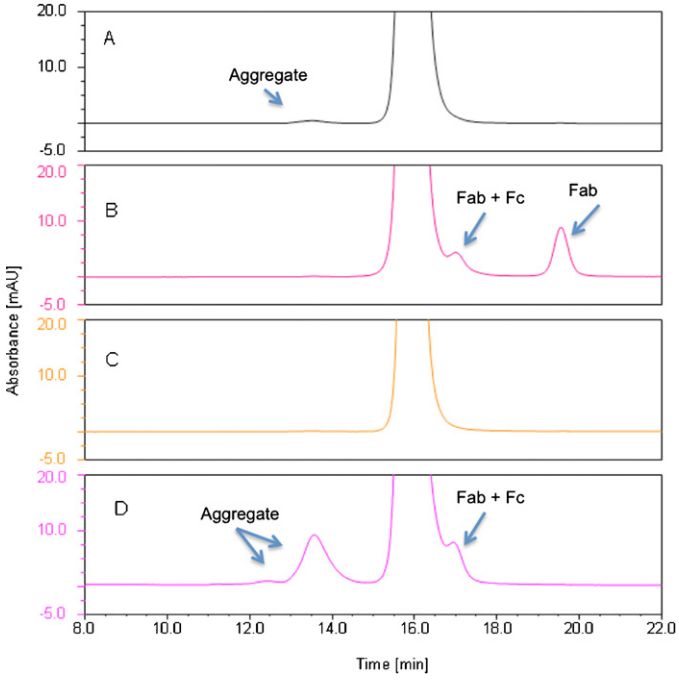

Fig. 11. SEC chromatograms of representative fractions from the acidic variant, main peak, and basic variant regions of the cation exchange displacement train (B, C, and D respectively). (A) Reference SEC chromatogram for the MAb that represents the load to the cation exchange displacement column.

antigen binding, they have been reported to influence FcRn binding and they could potentially affect the half-life of the antibody [22]. By utilizing displacement chromatography, we achieved the enrichment of such species and have identified where in the HPLC gradient cation-exchange assay this variant would appear if it were ever to increase (*e.g.* due to transient oxidation damage during in process or shelf storage). The enrichment achieved during the displacement separation also allows further study of this variant through binding and potency assays.

3.3. Additional characterization of the displacement train

As discussed above, the MAb charge variants are customarily defined by the elution position in the HPLC gradient cation-exchange assay. Apart from the charge variants defined by this assay, other types of variants within the displacement train may be identified by orthogonal assays. To this end we analyzed the displacement train with additional assays to explore the possibility of other type of variants being resolved in the displacement separation.

3.3.1. Size exclusion chromatography (SEC) analysis of the displacement train

SEC has been widely used for soluble antibody aggregate and fragment analysis. Here, SEC was carried out to evaluate size heterogeneity across the cation exchange displacement train. The SEC chromatograms with representative acidic, main and basic displacement fractions were compared with loading material as shown in Fig. 11. The reference MAb (displacement load material) had approximately 0.3% soluble aggregate and less than 0.1% fragment. The fragment species were enriched to 5.9% in the acidic fraction of the displacement train (a 20-fold enrichment) and were reduced to <0.1% in the main peak fraction. Fragments were also enriched in the basic fraction of the displacement train. The fragments identified in the acidic fraction were both Fab and desFab (Fab–Fc) species while the fragments identified in the basic fractions were mainly desFab species.

The aggregate species were enriched to up 10% in the basic fraction of the displacement train compared to 0.3% in the load material (33-fold enrichment) indicating that these particular aggregate species had a higher affinity to the cation-exchange resin than the smaller isoforms. For the main peak region of the displacement train, both the aggregate and fragment level was reduced to below 0.1%, providing an essentially pure sample of MAb monomer (>99.9%). Coupled with high protein concentration in the displacement train, cation-exchange displacement appears promising for the enrichment of large aggregates or the production of pure MAb monomer.

3.3.2. Glycan analysis of the displacement fractions

Glycosylation is an important attribute for therapeutic proteins and glycan variants have previously been enriched by multi-lectin affinity chromatography [23]. We analyzed the cation-exchange displacement train to verify whether glycan distributions remained consistent across the isolated acidic, main and basic variant groups. Key glycans such as sialic acid (NANA), afucosylation (G0-F), G0-1,

Table 2
Glycan analysis results (%) for selected acidic, basic and main peak fractions of the displacement train. Enrichment factor of the glycan from the load material is indicated below each percentage value. 1× indicates no enrichment.

	NANA	G0-F	G0-1	G0	G1/G1′	G2
MAb load	0.34	1.66	1.33	72.20	21.52	1.93
Displacement fractions						
Acidic fraction 1	0.52 (1.5x)	0.79 (0.5x)	1.26 (0.9x)	71.17 (1.0x)	22.63 (1.1x)	2.40 (1.2x)
Acidic fraction 2	1.06 (3.1x)	0.85 (0.5x)	1.82 (1.4x)	71.05 (1.0x)	21.97 (1.0x)	2.42 (1.3x)
Main peak fraction	0.15 (0.4x)	2.05 (1.2x)	2.04 (1.5x)	74.73 (1.0x)	18.85 (0.9x)	1.39 (0.7x)
Basic fraction 1	0.17 (0.5x)	1.86 (1.1x)	1.96 (1.5x)	74.37 (1.0x)	19.03 (0.9x)	1.58 (0.8x)
Basic fraction 2	0.16 (0.5x)	1.98 (1.2x)	1.76 (1.3x)	73.17 (1.0x)	20.35 (0.9x)	1.65 (0.9x)

G0, G1/G1′ and G2 are listed in Table 2 for acidic, basic and main peak fractions of the cation exchange displacement train. Other minor glycan distributions were below 1% in abundance and are not shown here. Enrichment of the glycans from the load material is also listed in the table below each glycan percentage. Enrichment above 2.0x and below 0.5x was considered a meaningful enrichment for low abundance glycans but not necessarily a substantial enrichment. Overall, the distribution of glycans did not appear to vary *substantially* across the displacement train, indicating that glycan heterogeneity is mostly independent of charge heterogeneity as defined by the cation-exchange HPLC assay. Closer analysis of the distribution of NANA indicated a small but meaningful (3×) enrichment of the low abundance NANA in the selected acidic displacement fractions. Such enrichment would be expected due to the acidic nature of the sialic acid modifications, however this enrichment was not substantial since the levels increased from 0.34% in the load material to 1.06% in the enriched fraction and was thus still considered a low abundance glycan. The distribution of G0-F appeared slightly lower (0.5× enrichment) in the selected acidic displacement fractions compared to the reference MAb and the other main peak and basic fractions. The reason for G0-F differences in the acidic fraction is unclear. The changes in G0-F percentage may not seem substantial in the acidic fraction, however it has been shown that even small changes in G0-F can have a significant impact on ADCC activity [24].

4. Conclusions

In this paper, we demonstrate the value of including displacement chromatography in the MAb characterization toolkit and highlight the potential to greatly enhance the information available about complex biological therapeutics. As a preparative technique, cation exchange displacement chromatography allows significant enrichment of charge variants with high purity and recovery. Our current technique allows isolation and enrichment of highly pure (>90%) charge variants from a 200 mg MAb sample in a single displacement run using two small semi-preparative columns in tandem. The throughput of this technique is approximately 10 times faster than standard preparative techniques for isolation these variants. Utilization of this technique can lead to significantly faster product characterization timelines and will most certainly lead to enhanced product understanding, the latter of which is essential for a successful QbD characterization package. In addition, the higher throughput of the displacement chromatography method makes it ideal for isolating charge variants for use in *in vivo* studies. The requirements for sterility and endotoxin are greatly increased in this regard and the high throughput and reduced sample manipulation of the displacement method increases the chance of success as compared to a traditional HPLC gradient approach for isolating these variants.

In an analytical capacity, we show how displacement chromatography can reveal the presence of trace variants. Identification of the methionine oxidized trace variant in the MAb reference material has several implications. While a variant in such a minute level is typically not a cause for concern, the identification of the presence of this trace variant in the drug substance elucidates the location of elution of this variant on the cation exchange gradient assay. In addition, the enrichment and further analysis of this variant further elucidates the location of residues susceptible to oxidation for this MAb.

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