**ABSTRACT**

Isolation and characterization of monoclonal antibody (mAb) variants to understand the impact of their structure on function is a typical activity during early-stage candidate selection that contributes to derisking clinical development. In particular, efforts are devoted to characterizing oligomeric variants, owing to their potential immunogenic nature. We report here a mAb variant consisting of a canonical mAb monomer associated in a non-covalent fashion with an antigen-binding fragment (Fab) arm amputated from its Fc domain. The truncated heavy chain is encoded in the cell line genome and is the likely product of a genomic recombination during cell line generation. The addition of the Fab arm results in severe loss of potency, indicating its interaction with the Fab domain of the monomer. The presence of such a variant can easily be mitigated by an adequate purification step.

**Introduction**

Isolation and characterization of monoclonal antibody (mAb) variants are performed prior to entering clinical development to ensure the selection of the therapeutic candidate with the best biochemical and biophysical profile, which should enable rapid development and subsequent approval. Understanding the nature of the variants is the first step towards assessing the impact of the molecular or structural variations on function. The knowledge of the structure-function relationship contributes to the development of the most appropriate manufacturing process and quality control strategy, and ultimately ensures the efficacy and safety of the therapeutic protein product to be delivered to patients.

Typical modifications encountered in complementary-determining regions, scaffold and conserved domains of light and heavy chains of IgG mAbs have been reported in numerous reviews. Such modifications include but are not limited to N- and O-glycosylation, oxidation, deamidation, isomerization, glycation, and O-glycosylation, oxidation, deamidation. Departures from the intended amino acid sequence may result in severe impact on potency through the disruption of the mAb target antigen engagement, increased or decreased affinity for complement proteins and the different Fc gamma receptors, reduced half-life duration, and increased aggregation propensity.3,4,6,8,9

As is the case for the variations affecting the primary sequence, the formation of different oligomeric forms reflective of a change in the canonical quaternary arrangement of the mAb can also result in the alteration of the function, stability, immunogenicity and overall safety of the therapeutic mAb.10-12 Although clinical evidence for the immunogenic potential of therapeutic antibodies aggregates is scarce, the correlation between IgG high order structures and adverse events in human is well documented.11,12 While the exact mechanisms leading to immune reaction are difficult to establish, the ability of IgG high order structures to mobilize and interfere with both the innate or acquired arms of the immune system is known.11,12 In addition to immunogenicity concerns, departure from the canonical IgG monomeric structure may alter the molecule’s function. In particular, mAb dimers were shown to have increased affinity for Fc gamma receptors.13 Likewise, the alteration of the mAb canonical quaternary structure through oligomerization, loss or addition of one or more light chains was shown to be deleterious to a variant’s ability to recognize its specific target antigen.14,15

As part of the effort to list the critical quality attributes of a recombinant IgG produced in Chinese hamster ovary (CHO) cells, we biochemically and functionally characterized quaternary structure variants separated by SEC-HPLC.

**Results**

**Detection of a 200 kDa species in a recombinant mAb by SEC-HPLC**

Analysis by size exclusion HPLC (SEC-HPLC) of purification intermediates during process development showed the presence of a well-resolved peak eluting between the peaks typically attributed to the mAb in its monomeric and dimeric forms (Fig. 1A). Analysis of the sample by in-line multi-angle light scattering (MALS) after separation by SEC-HPLC confirmed...
the monomer and dimer peak assignments (Fig. 1B). It also attributed an approximately 200 kDa molecular mass to the additional peak (Fig. 1B). In order to characterize this unexpected species, we fractionated the sample into three pools: Main peak (monomer), 200 kDa fraction, and a pool including the mAb dimers and larger molecular weight species. The fractions were 99%, 96% and 88% pure, respectively, after reinjection on the SEC-HPLC column (Fig. 1C).

The 200 kDa species is composed of a mAb and a 50 kDa subunit

The purified fractions were first analyzed by non-reduced capillary electrophoresis-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (CE-SDS). The 200 kDa material migrates as two peaks, one aligned with the monomer in the control main peak fraction (Fig. 2A). The second one had the electrophoretic mobility similar to that of a free heavy chain. The dimer fraction was, as expected, composed of mAb monomer and possibly covalent aggregates not dissociated by the action of SDS. These results were confirmed by non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2B). The 200 kDa sample did run as two species: one with a mobility of a ~150 kDa protein identified as the mAb monomer and a second consistent with a protein of molecular mass estimated at 50 kDa.

Lastly, the purified fractions were deglycosylated and analyzed by non-reduced LC-MS (Fig. 2C). The antibody monomer was detected in all samples (145,111 Da in the Monomer fraction, 145,113 Da in the 200 kDa fraction for a theoretical value of 145105 Da, Table 1). In addition to the monomer, the 200 kDa fraction contained a second species with an observed mass of 49,877 Da, a value consistent with the non-reduced CE-SDS and SDS-PAGE results (Table 1). When the same experiment was repeated without deglycosylation prior to LC-MS analysis, the masses for the monomer in the 200 kDa fraction and control samples increased to reflect the presence of the expected major G0 and G0F glycoforms. In contrast, the mass of the additional 50 kDa component in the 200 kDa fraction remained unchanged, suggesting the absence of N-glycans.

The CE-SDS, SDS-PAGE and mass spectrometry data all indicate that the 200 kDa species is the result of a non-covalent association of a 150 kDa monomer and a non-N-glycosylated 50 kDa protein.
The 50 kDa protein is composed of a LC and an unknown polypeptide

Next, we set out to determine the nature of the 50 kDa component. The samples enriched in 200 kDa species, dimer, and control were tested by reduced CE-SDS (Fig. 3A). After reduction, all samples showed species with the electrophoretic mobility of the light and heavy chains. The 200 kDa fraction sample had a significant shoulder on the tailing end of the light chain, suggesting the co-migration of different species with a size close to that of a light chain. In addition, the light chain to heavy chain ratio was found to be markedly higher in the 200 kDa fraction sample when compared to the Monomer sample and starting material.

Next, we performed amino terminus sequencing by Edman degradation after separation on a non-reduced SDS-PAGE and blotting on a membrane. The determined sequence of the band migrating at 50 kDa aligned perfectly with that of the amino terminus of the light chain. While this result is consistent with the CE results and confirms the presence of a mature and adequately processed light chain, it does not rule out the presence of other polypeptides blocked at their amino terminus.

Last, we analyzed the purified fractions by reversed phase HPLC after digestion at the hinge with IdeS protease and reduction (Fig. 3B). For all samples, the profiles showed the expected peaks assigned to light chain, the carboxy terminus of the heavy chain clipped in the lower hinge region (i.e., Fc/2) and its heavy chain amino terminus fragment counterpart (Fd') and its variants. However, one peak was present only in the 200 kDa fraction sample. Measurement of the molecular mass of the unknown peak by in-line LC-MS showed a species with a mass of 25804 Da (Fig. 3C). We surmised that this species may originate from a light chain with partly processed secretion leader sequence blocked at the amino terminus, or from a heavy chain that would be truncated near the mAb hinge sequence. However, no truncated heavy chain or incompletely processed light chain could account for the 25804 Da detected mass.

Reduced CE-SDS, amino terminus sequencing and LC-MS showed that the unique 50 kDa component present in the 200 kDa fraction is the result of the disulfide-mediated covalent association between a mature LC and an unknown polypeptide with a 25804 Da mass.

Identification of the unknown polypeptide

Tryptic peptide mapping was performed on the 200 kDa species sample and, as control, on the monomer and dimer fraction samples (Fig. 4A). The map of the digest of the three samples shared the vast majority of the identified peptides, albeit with evident differences in peak intensity and recovery. This observation lent credence to the notion that the unknown peptide is highly related to the mAb LC or HC polypeptides, and ruled out the possibility of an adduct with a CHO host cell protein.

De novo MS/MS sequencing of one of three peptide mapping peaks present only in the 200 kDa fraction sample and absent from the Main peak fraction sample revealed an amino acid sequence with no homology to the mAb LC or HC (Fig. 4B). However, the putative peptide eluting as this unknown peak had a sequence matching that of a gene product obtained by the in-frame translation of an intron located between the CH1 domain and hinge exons (Fig. S1). The CHO cell line used to produce the mAb was indeed obtained via the insertion in the genome of a DNA

Table 1. Molecular mass of the major components in the purified fractions.

| Purified fraction | Theoretical Mass (Da) | Monomer | 200 kDa | Dimer |
|-------------------|-----------------------|---------|---------|-------|
| Intact Ab<sup>+</sup>| 145105                | 145111  | 145113  | 145114|
| Unknown           | N/A                   | 49877   | —       | —     |

<sup>+</sup>With HC N-terminal pyro-E and without C-terminal Lys.

**Figure 3.** The 50 kDa is composed of a light chain (LC) and an unknown polypeptide. (A) Overlay of reduced CE-SDS traces of the monomer fraction (top trace), the 200 kDa species and dimer fractions (middle traces), and blank injection control (bottom trace). (B) Overlay of IdeS digested and reduced RP-HPLC traces of the monomer fraction (top trace), the 200 kDa species (middle trace), and dimer fractions (bottom trace). The peaks corresponding to LC, Fc/2 and Fd' in the three samples are labeled. The peak unique to the 200 kDa species fraction is indicated with an asterisk. (C) Deconvoluted mass spectrum of the peak unique to the 200 kDa species fraction showing a mass of 25804.3 Da.
containing light chain and heavy chain gene cassettes organized in a manner similar to that of a B cell (Fig. 5C). Two other peptides were subsequently identified in the tryptic map that matched different portions of the same intron sequence, thus confirming our hypothesis. The sequence of the peptide appended to the CH1 domain, leading to the truncation of the heavy chain, was confirmed by peptide mapping using LysC instead of trypsin (Fig. S2). A unique peptide was found in the map of the 200 kDa species sample compared to control. This peptide had a mass consistent with the predicted sequence, included 2 amino acids of the CH1 domain and 30 amino acids of the translated intron, and included all the intron amino acids identified by tryptic peptide mapping (Fig. 4C). This result showed we had identified the complete sequence appended to the CH1 domain. This was further confirmed by the fact that the predicted mass of the heavy chain truncated within the translated intron following the CH1 domain (25804.98 Da) was in good accord with the 25804 Da mass observed in the reduced Ides digest LC-MS experiment (Fig. 3C).

Lastly, to fully understand the origin of the truncated heavy chain species, targeted locus amplification (TLA) sequencing was utilized to map the junctions between sequences corresponding to two adjacent transgene-containing vector copies. The TLA sequencing method relies first on the generation of a library of transgene and immediately adjacent host DNA circles produced via targeted crosslinking, digestion, re-ligation, and PCR amplification using a primer pair complementary to a short transgene specific sequence. Library deep sequencing and mapping of the resulting DNA sequence reveals the different transgenes integration sites in the host genome, transgenes vector sequence, and adjacent vector-vector fusions without prior knowledge of the integration site and possible mutations in the transgene sequence. This methodology is more efficient than the whole genome sequencing or capture-based targeted re-sequencing approaches. Results showed most transgene copies to include all intended exons and introns, thus resulting in the expected light and heavy chain sequences and in the canonical IgG quaternary structure (Fig. 5C). However, two of the transgenes did include a heavy chain gene lacking the hinge,
C_{1\alpha}2 and C_{1\beta}3 exons and truncated shortly after the exon following the C_{\alpha}1 domain, implying the genetic origin of the 200 kDa species (Fig. 5C). The mechanism leading to the truncation of one heavy chain coding sequence is unknown, but, based on the result of the TLA sequencing, is likely due to DNA recombination events during cell line generation.

**The additional Fab is bound to the mAb Fab**

Next, we set out to gain a better understanding of the 200 kDa species quaternary structure. Analyses conducted on the purified 200 kDa species showed the light chain and truncated heavy chain addition to be non-covalently associated to the mAb (Fig. 2, Fig. 3). However, it did not resolve whether the interaction was Fc or Fab mediated. To resolve this point, we compared the relative potency of the purified 200 kDa fraction and compared it to that of the purified monomer peak control fraction. Results showed that the 200 kDa had less than half the relative potency of the control monomer peak sample (Fig. 5A, Table 2). This result suggested that the 50 kDa unit is associated with the mAb Fab arms. To confirm this result, IdeS-digested samples were analyzed by SEC-UPLC with in-line MALS detection (Fig. 5B). The chromatogram of the hinge-digested 200 kDa purified sample had two major peaks. One corresponded to a species with a mass of about 50 kDa and the same retention time as that of a peak present in the purified monomer peak control sample. This peak was attributed to the Fc domain. The second peak had an observed mass of about 150 kDa, but did not align with the \(50 \text{kDa} \times 2\) peak observed in the control sample. This peak also had a retention time bracketed by the undigested 200 kDa control on the high molecular mass side and the regular undigested mAb monomer in the purified monomer peak control sample. Based on the potency and SEC-MALS data, we concluded that the interaction between the 50 kDa light chain/truncated heavy chain adjunct is mediated by the mAb Fab domain (Fig. 5C). The exact nature of the interaction at the residue level was not further investigated.

**Removal of the 200 kDa during purification**

A purification process that removes the 200 kDa impurity was then developed. The purification process change included chromatography over a strong cation exchanger in sodium phosphate and NaCl buffer for binding and elution over a step gradient of five column volumes. Purification intermediates were analyzed by SEC-MALS (Fig. 6A) and regular SEC-HPLC (Fig. 6B). As earlier, the 200 kDa species was detected in the first intermediate after capture on a Protein A column (Fig. 6A). After the modification of the purification process, chromatography over a strong cation exchanger was able to effectively clear the 200 kDa species. SEC-MALS and SEC-HPLC show the absence in the post-purification sample of the peak assigned to the 200 kDa species and eluting between the monomer (\(\sim 150 \text{kDa}\)) and dimer (\(\sim 300 \text{kDa}\)) (Fig. 6A and B). SEC-MALS also showed the presence of a species eluting after the main peak. This peak is not related to the 200 kDa species and corresponds to a species with an assigned mass very close to that of the mAb monomer. The nature of this species has not been elucidated at this point.

**Discussion**

Here, we purified, characterized and identified a new mAb-related structure observed in SEC profiles from a preparation of an early-stage recombinant mAb produced by a CHO cell line. The variant consisted of a canonical mAb monomer associated in a non-covalent fashion with a Fab arm amputated from its Fc domain. The truncated heavy chain is encoded in the cell line genome. This variant is the product of a DNA

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Table 2. Relative potency of the purified fractions.

| Purified fraction | Monomer | 200 kDa | Dimer |
|-------------------|---------|---------|-------|
| Relative potency  | 114%    | 23%     | 199%  |

1 Relative potency is expressed as the ratio of the potency of a give sample dived by that of a reference sample used in the assay.
recombination event that occurred during the insertion of the transgene construct and amplification steps and was propagated during cell line selection. While this particular modification has not been described to date, it adds to the long list of variants described in the literature for the many mAbs that are approved or under clinical development.1-3

Modifications resulting from undesired genetic events during transfection and amplification are not uncommon and have previously been reported in the literature.15-22 Perhaps the most frequently described is the alteration of a nucleotide in the translated portion of the coding DNA, resulting in mis-sense or non-sense mutations and eventually in the insertion of an erroneous amino acid in the recombinant protein, premature translation termination, or carboxy terminus extension.18-21 Although not directly the result of a genetic alteration, but rather of an inadequate codon optimization, frame shifts at slippery codons have also been reported to be the cause of aberrant sequences and early translation termination.22

When not removed during purification, the sum of these local departures to the intended sequence or structure, whether the result of the modification of an amino acid side chain through post-translational modification or from genetic origin, constitutes the micro-heterogeneity of the therapeutic mAb. Each purified therapeutic mAb should therefore be regarded as a collection of closely related sequences rather than a perfectly homogenous structure. Any of the variants that affect potency or safety, increasing during storage or compromising the stability of the molecule, are considered a product-related impurity.23

Modifications occurring post-translationally are the direct reflection of the chemical-physical stability of the clinical candidate selected for development. Carefully crafted and executed candidate selection and developability studies can detect most of the liabilities and lead to their removal before the candidate enters clinical development or, at minimum, inform the analytical monitoring and control strategy.8,24 In contrast, protein variants occurring as a result of genetic rearrangements or mutations are a property of the cell line selected. These kinds of modifications can be relatively easily detected and alleviated by enabling product quality assessment during clonal selection or mitigated by the development of an adequate purification process.

**Material and methods**

**mAb Production and Purification**

The mab is a recombinant humanized IgG produced using a CHO dhfr− cell line and a standard fermentation process. Cell culture harvests were clarified by centrifugation and filtration followed by capture on a Protein A column. Elution from the Protein A column was performed using a sodium acetate buffer as per standard industry practices. The mAb was further purified by two subsequent polishing steps including cation exchange and anion exchange/mixed mode. Purification intermediates and their corresponding matrices were provided to ensure suitability of the analytical tests. The fully purified material was provided in a sodium phosphate, sodium chloride and PS80 formulation.

**SEC-HPLC**

The isolation of mAb high molecular weight and main peak species was carried out on an Agilent 1260 HPLC equipped with a fraction collector. Approximately 1 mg of mAb was eluted isocratically at 0.5 mL/min on a Tosoh Biosciences G3000SWxl column (7.8 mm ID x 30 cm), using a mobile phase consisting of 0.2 M potassium phosphate, sodium chloride and PS80 formulation.

**SEC-MALS**

To determine average molar mass of mAb size variants, a Waters Acquity UPLC system was used to isocratically elute 20 μg of mAb at 0.1 or 0.2 mL/min on a Waters SEC 200 BEH column (4.6 mm ID x 300 mm), using a mobile phase consisting of 0.2 M potassium phosphate, 0.25 M potassium chloride, pH 6.2, and UV detection at 280 nm. Fractions from multiple purification cycles were pooled and concentrated to > 1 mg/mL using 10,000 MWCO centrifugal filter units, buffer exchanged for storage. Purity was verified by re-injecting 25–50 μg onto the same column.
**CE-SDS**

Molecular weight-based separations of mAb fractions were performed on a Beckman PA800 plus using the IgG Purity and Heterogeneity Assay Kit. After diluting with SDS sample buffer, samples were either reduced with 5% β-mercaptoethanol or alkylated with 12.5 mM iodoacetamide, using injection times of 30 seconds and 40 seconds, respectively. Detection wavelength was set at 214 nm.

**SDS-PAGE**

Non-reducing SDS-PAGE separation was performed using the NuPAGE pre-cast gel system (Thermo Scientific). Ten μg of sample was dissolved in LDS sample buffer with 10 mM dithiothreitol and heated (70°C, 10 minutes), loaded onto a NuPAGE 12% Bis-Tris gel, and separated with a MOPS running buffer at 200V for 50 minutes. The gel was stained with Ponceau stain.

**Trypsin and Endo Lys-C peptide mapping**

Peptide mapping was carried out on Dionex UltiMate 3000 UPLC system connected in line with an Orbitrap Elite mass spectrometer (Thermo Scientific). Samples were denatured in Tris pH 8.0 buffer containing 6 M guanidine chloride, reduced with 5 mM TCEP, then alkylated with 25 mM iodoacetamide in the dark. Excess reagent was removed using 10,000 MWCO centrifugal filter units (Millipore) and buffer exchanged into 50 mM ammonium bicarbonate pH 7.8. Samples were incubated with either trypsin (Promega) or Endo Lys-C (Roche) at a ratio of 1:20 (w/w) for 15 hours at 37°C. Peptide separations were then performed on a Waters BioSuite C18 PA-A 3 μm column (2.1 mm x 150 mm) at 40°C using mobile phases consisting of 0.1% formic acid (FA) in water (solvent A) and 0.1% FA in acetonitrile (solvent B). Flow rate was set at 0.3 mL/min and a linear gradient of 0–40% B over 45 minutes was used for elution and monitored at 214 nm. Orbitrap MS parameters were as follows: mass range, 200–2000 m/z; CID normalized collision energy, 35%. Raw MS and MS/MS data were processed with PepFinder v2.0 (Thermo Scientific).

**Intact and IdeS-digested MS analysis**

Reverse phase MS analyses of mAb fractions were performed on a Waters Acquity UPLC system connected in line with a Waters Zevo G2 mass spectrometer. Zero charge deconvolutions were carried out in MassLynx v4.1 (Waters) with the Waters Zevo G2 mass spectrometer. Zero charge deconvolution on a Waters Acquity UPLC system connected in line with a Reverse phase MS analyses of mAb fractions were performed intact and IdeS-digested MS analysis with PepFinder v2.0 (Thermo Scientific) collision energy, 35%. Raw MS and MS/MS data were processed with b (4-PL) curves in SoftMax Pro software to determine equivalency and parallelism. The relative potency is calculated from a constrained 4-PL curve by dividing the half maximal effective concentration (EC50) of the reference standard by the result is reported as the relative potency of the sample compared to the reference standard.

**mAb functional analysis**

The binding activity of the mAb to its antigen is determined by direct enzyme linked immunosorbent assay (ELISA). In the assay, serial dilutions of the mAb reference standard, control, and sample are added to a microtiter plate coated with recombinant human target antigen (R&D Systems Cat #2639-A0) and incubated at room temperature for 1 hour. The bound mAb on the plate is detected with mouse anti-human IgG antibody conjugated to horseradish peroxidase, (HRP), Southern Biotech, Cat# 9190-05. After the second incubation and wash, the HRP substrate, 3,3',5,5'-tetramethybenzidine is added to develop a soluble blue color. The colorimetric reaction is quenched with hydrochloric acid and the optical density is measured at 450 nm wave length by the microplate reader. The dose-response curves are analyzed with unconstrained four-parameter logistic fit (4-PL) curves in SoftMax Pro software to determine equivalency and parallelism. The relative potency is calculated from a constrained 4-PL curve by dividing the half maximal effective concentration (EC50) of the reference standard by the result is reported as the relative potency of the sample compared to the reference standard.

**TLA sequencing**

Preparation of the samples for TLA was performed as per established procedure. In summary, the recombinant CHO cell line population expressing the humanized IgG was crosslinked using formaldehyde and DNA was digested with NalIII. The samples were ligated, crosslinking was reversed, and the DNA was purified. DNA molecules were cut with NspI and ligated to obtain circular chimeric DNA molecules that were then PCR amplified. Primer sequences were complementary to the transgene vector sequence and included the following sets (5’ to 3’): SET1 Forward CAGAGCTCGTTTATGGAAC; SET1 Reverse CCGAGTTGT-TACGACATTTT; SET2 Forward TTTGCCTAAAGCATT- TACGACATTTT; SET2 Reverse AAGAATGGCCACGTCATC. Eight 25-μL PCR reactions, each containing 100 ng template, were pooled for sequencing. Illumina NexteraXT NGS library preparations were performed according to manufacture's protocols. TLA libraries were sequenced on the Illumina MiSeq platform pooling ~20 libraries per V2 PE150 sequencing run yielding on average 1 million reads per library. Reads were mapped using split-read aware alignment with the Burrows-Wheeler Aligner (BWA) mapping software version 0.6.1-r104, settings: bwasw » 7. Paired ends were treated separately in the general analysis. The data were aligned to the CHO K1 and hamster DNA sequence publically available. The resulting BAM files were analyzed using Integrative Genomics Viewer.
Abbreviations

CE, capillary electrophoresis
CHO, Chinese hamster ovary
HC or LC, heavy or light chain;
MALS, multi-angle light scattering
SDS PAGE, sodium docecyl sulfate-polyacrylamide gel electrophoresis
SEC, size-exclusion chromatography.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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