Monoclonal Antibody Aggregation in Cell Culture: Aggregate Characterization and Protein Stability Analysis

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MONOCLONAL ANTIBODY AGGREGATION IN CELL CULTURE: AGGREGATE CHARACTERIZATION AND PROTEIN STABILITY ANALYSIS

by

Elisabeth Emily Russell

B.S./M.S., Tufts University, 2003

A thesis submitted to the
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written by Elisabeth Emily Russell
has been approved for the Department of Chemical and Biological Engineering

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Date_________________

A final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
Protein aggregates represent a safety, immunological and stability concern in therapeutic protein formulations. Aggregates may be formed at any stage during the manufacturing process, including during cell culture. In mammalian cell culture, the proteins that are produced typically are incubated in the cell culture medium at relatively high temperatures (e.g. 36 °C) for prolonged periods of time (1-2 weeks). To investigate potential mechanisms by which this incubation might generate protein aggregates, we examined the stability of a model monoclonal antibody (mAb) under cell culture conditions, but in the absence of cells. A step-wise approach was used to eliminate factors that would be causing protein aggregation.

Results show that protein aggregation is mediated by the presence of extracellular monoclonal antibody light chains. This result was corroborated through aggregate characterization showing that a cell lines aggregation propensity correlated with the light chain content of HMWS.
Dedication

To my friends who have been my biggest cheerleaders: Pike’s Peak Marathoner, Rocky Mountain Heli-skier, Southern Belle, Big Sky Cowboy, N’Sync Fanatic, Whisky Aficionado, No Sheep in Mammoth Party Animal, Beautiful B and the Teamies.
Acknowledgments

Lesson 1: the water in the water bath is not all that clean

September 2011 was the first vial thaw and the first lesson I learned in aseptic lab technique. Thanks to Lisa and Suzy for getting me straightened out and showing me how to not contaminate my cell culture flasks. I wouldn’t have made it much past that day without your teachings.

Since that first vial thaw there have been more lessons learned, mistakes made, late night discoveries and moments of success none of which would have been possible without an immense support network helping me move forward. Thank you to my committee and fellow graduate students in the Randolph lab for asking all the right questions and challenging my ideas. Your involvement enabled me to efficiently direct my research and ultimately piece together part of the story behind protein aggregation in cell culture.

Thank you to everyone that supported my lab work and being patient as I figured out how to best get work done without totally disrupting the normal lab operations. Thank you to my colleagues at Amgen for engaging conversations and unwavering support. A special thanks to Chris Crowell…for pushing me to take another pass at getting into graduate school. Lesson learned here: don’t use the equivalent of a Crayola crayon to fill out your graduate school application.

Thank you to my parents, Andrew and Karen for all encouragement and putting up with the background noise that came with my ups and downs in the last four years (or 30+ years depending on who you ask).
Lastly, I’d like to thank Ted. Thank you for making every discussion a lesson in motivation. There wasn’t a time that I didn’t walk out of your office full of excitement to do more experiments or bury myself in literature reviews. You had to have known you succeeded when I started loving to run gels.
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Chapter 1: Introduction

Monoclonal antibodies (mAb) represent an important class of therapeutic agents. In 2014, there were an estimated 36 monoclonal antibodies in Phase 3 clinical trials for the treatment of a variety of human diseases including cancer, inflammation, metabolic disorders and bone disease [1]. One critical quality attribute of these therapeutic agents is the concentration of high molecular weight species (HMWS) and aggregates, where HMWS are classified as a subgroup of aggregates comprised of dimers and larger oligomers. The criticality of HMWS concentration in a drug product is based on the potential immunogenicity of aggregates [2, 3]. Due to the safety risk associated with HMWS regulatory agencies expect drug applications to include data that shows acceptable HMWS concentrations in the product and robust clearance of HMWS through the purification manufacturing process. The FDA assessment of the immunogenicity risk associated with protein products was recently released as a “Guidance for Industry” publication whereby it is stated that “It is critical for manufacturers of therapeutic protein products to minimize protein aggregation to the extent possible.”

As monoclonal antibody processes have developed, increased supply requirements and desire to reduce manufacturing costs have resulted in higher protein concentrations. With higher protein concentrations, a higher propensity for HMWS in the cell culture process has been observed. This correlation is not unexpected given the extensive work showing an increase in aggregation rates with higher protein concentrations [4-8]. Increased HMWS decrease product yield and have the potential to increase complexity of downstream processing, impacting resin choice and required number of purification steps. Due to the impact on the manufacturing process and potential to impact product safety and stability, understanding HMWS formation mechanisms, with a goal of controlling aggregation in cell culture process would enable
development of higher yield processes producing higher quality products. This chapter will discuss topics of relevance to protein aggregation in cell culture such as aggregation mechanisms, intracellular monoclonal antibody formation, cellular stress response and clone selection.

1.1. Approach and objectives

Studies were designed to demonstrate that aggregation was occurring extracellularly and identify factors that support protein aggregation. To investigate extracellular protein aggregation a stepwise approach was taken to eliminate potential factors. The list below categorizes the factors into three groups:

1) Aggregate characteristics: free thiol content, glycosylation, extracellular light chain levels

2) Chemical: osmolality, pH, spent media components, redox potential, protein concentration

3) Physical: agitation and temperature

Studies were performed using three Chinese hamster ovary (CHO) cell lines. Two were clonal cell lines that produced the same monoclonal antibody but one cell line produced twice the level of protein aggregates. This contrast in aggregation levels allowed for side-by-side comparison of results to identify correlating factors to aggregation propensity. The third cell line was a null cell line that did not express the protein of interest and was used as a negative control for studies. The objectives of this research were to:

- Determine factors that correlate with extracellular protein aggregation in the absence of cells using a shake flask model (Chapter 3 and 4)
- Characterize protein aggregates to determine correlating product quality characteristics with protein aggregation (Chapter 4 and Appendix A)

- Demonstrate that aggregation can be modulated based on contributing factors or proposed pathway (Chapter 4)

- Investigate the impact that protein-A chromatography purification has on the HMWS content of cell culture samples (Chapter 2). Establish a robust analytical method for quantifying HMWS in cell culture samples without the use of protein-A chromatography purification (Appendix B)

1.2. Aggregation mechanisms

Aggregation pathways can be defined by the initial state of the protein, intermediate formation and the end state of the protein. Initial states include native, degraded, modified structure, partially unfolded or fully unfolded [5]. End states include dimers, oligomers, molten globules and precipitates. Wang et al. described the major pathways as follows [9]:

- Aggregation through unfolding intermediates and unfolded states

- Aggregation through protein self-association or chemical linkages

- Aggregation through chemical degradations

The first pathway is initiated under conditions that promote protein unfolding. This can be triggered by changes in the environment surrounding the protein that weaken the intramolecular bonds [10]. As the protein unfolds hydrophobic regions can be exposed. These protein conformations are energetically unfavorable and as a result can cause aggregation as hydrophobic regions bind to other hydrophobic regions on neighboring proteins to move to a more stable energy state [11]. Monoclonal antibodies can aggregate via partial unfolding. Due to
the protein structure being comprised of four major domains there are variations on the unfolding pathway leading to an intermediate [12].

Aggregation through self-association is predominately due to colloidal instability in the protein. This instability can be estimated based on the second osmotic virial coefficient that reflects the attraction/repulsion forces between a protein and itself [13]. A positive value indicates there are net repulsive forces between molecules while a negative result suggests net attractive forces. Self-association of an IgG antibody was shown to occur through electrostatic and hydrophobic interactions suggesting that some of the aggregation pathways do not need an unfolding intermediate to aggregate [14, 15].

Aggregation through chemical linkages includes pathways where a protein disulfide bond is reduced and the exposed thiol group on the cysteine is able to bind to neighboring free-thiol groups [16]. Monoclonal antibodies can form covalent aggregates via disulfide bond formation from the presences of exposed free-thiol groups [17]. This mechanism could occur during protein folding prior to the completion of disulfide bond formation or under partial unfolding conditions. Monoclonal antibodies have been shown to aggregate under a wide variety of conditions and through various pathways [18]. The aggregates formed in cell culture can be formed intracellularly and secreted or extracellularly. Intracellular aggregation can occur during the protein folding process as the protein conformation is not stable. Examples include aggregation in the endoplasmic reticulum due to the redox potential and low pH conditions [5, 19, 20]. Additionally, incompletely assembled mAb can be present and are more prone to aggregation due to exposed regions and being in a conformationally unstable state relative to a native form.

Factors that could contribute to extracellular aggregation include solution pH, osmolality or ionic strength [5-7, 15, 21-23], media components or additives [5-7, 15, 24-29], solution
temperature [5, 7, 15, 30, 31], protein concentration [4-8], solution redox potential [20, 25] and presence of misfolded monomeric proteins or monomeric fragments [32-39]. Ultimately, there may be multiple extracellular factors that contribute to an overall increase in aggregate levels via various aggregation pathways.

1.3. Monoclonal antibody folding and assembly

Intracellular aggregation can occur due to environmental conditions that promote protein instability and due to the generation of folding intermediates that have exposed hydrophobic regions or incomplete disulfide bonds [40]. Monoclonal antibodies are comprised of four peptides namely, two light chains and two heavy chains that are linked by disulfide bonds. The folding process, as explained by Feige et al. starts in the endoplasmic reticulum [41]. The heavy chain starts folding with the assistance of a binding protein (BiP), and this is followed by heavy chain dimerization and disulfide bond formation. Concurrently, the light chain is rapidly folded. Two light chains and a heavy chain dimer bind which releases the BiP allowing the remaining heavy chain regions to complete folding. The remaining disulfide bridges are then formed. There is a quality control mechanism termed ER-associated degradation (ERAD) whereby incompletely assembled or folded antibodies are transported to the cytosol for degradation [42]. This process limits the number of misfolded or incomplete antibodies that are secreted. Protein glycosylation starts in the endoplasmic reticulum and for some proteins is necessary to mediate the folding process. The final glycosylation occurs in the golgi once the protein is completely folded [43].

Complete and proper disulfide bond formation is not needed for the mAb to be secreted [44, 45]. As a result incomplete mAb proteins can be released into the cell culture in the form of light
chain dimers (LC₂) or as a heavy chain dimer with a single light chain (HC₂LC) [33, 34]. Although heavy chains and heavy chain dimers are not expected to be secreted due to their interaction with BiP, some immunoglobulin allotypes have been shown to pass the quality control system and can be secreted as heavy chain dimers [46].

Once the mAb passes through the golgi it can be transported by cellular vesicles to the plasma membrane. At the plasma membrane interface the vesicle binds to the surface and releases the mAb into the extracellular medium.

1.4. Cellular stress response

Recent research has enabled the development of high productivity cell lines but with this designed change in cell mechanics is an increase in cellular stress [47, 48]. It has been found that stress on the cell can impact the quality of protein expression resulting in misfolded monomers. The unfolding protein response (UPR) is an intracellular mechanism that is triggered in response to cell stress that can be caused by peptide over expression and peptide accumulation in the endoplasmic reticulum [49, 50]. The activation of the UPR triggers an increase in production of specific host cell proteins such as BiP to help the cell process (i.e. fold, assemble and secrete) the excess peptides [51]. Additionally, the there is a down-regulation of protein synthesis to lower the intracellular peptide levels and restore homeostasis. Although this process exists as a preservation mechanism for the cell, under unmanageable processing conditions, such as over expression of a mAb, the cell malfunctions leading to intracellular aggregation or cell death [49, 50].
1.5. **Clone Selection and Model System**

Two clonal Chinese hamster ovary (CHO) cell lines were selected as the model system for assessing monoclonal antibody stability in cell culture. The clones represent unique cell lines that share the same DNA but differ in their protein expression based on the clone selection process. Clone selection is an early step in process development for a new therapeutic protein. The goal of clone selection is to identify a cell line that has the desired expression characteristics such as purity and concentration [36]. Multiple clones are screened for the desired characteristics and the preferred clones are taken forward for additional product/process development. Clones are generated by transfecting CHO cells with the plasmids containing the heavy chain and light chain DNA. The transfection process is random and the antibody coding DNA will randomly insert into the CHO cell DNA. Based on the random integration the resulting antibody expression will be modulated by where in the CHO DNA the plasmid DNA is inserted. In some cases the integration completely disturbs cellular function and the cells die. Alternately, the cells accept the integration and can express and assemble the antibody. Depending on the insertion, the antibody expression levels and quality will vary between clones. Selection of the two model clones was based on high titer (protein concentration) and high/low aggregation concentrations. Both clonal cell lines produce an IgG monoclonal antibody. The general structure of the protein is shown in [Error! Reference source not found.]. As is typical for an IgG, the antibody is composed of two light chains and two heavy chain peptides that together contain six inter-chain disulfide bonds and 12 intra-chain disulfide bonds. Additionally, there is one N-linked glycan on the heavy chain.
Figure 1. IgG monoclonal antibody diagram
Chapter 2: Impact of Protein-A Chromatography on the Aggregation State of Monoclonal Antibodies Isolated from Mammalian Cell Culture

2.1. Abstract

Direct analysis of protein aggregation within cell culture broths is challenging due to the complexity of these solutions. Unlike highly purified final drug products, mammalian cell culture broths contain background proteins, cell by-products and complex media components (e.g. amino acids, sugars, trace metals). To quantify the levels of aggregates of monoclonal antibodies (mAbs) in cell culture samples, affinity chromatography with protein-A resin is typically used to pretreat samples prior to analysis by size exclusion chromatography (SEC). Protein-A chromatography by itself may alter the distribution of aggregated protein species, either by dissociating existing aggregates or by fostering additional aggregation. The percentage of mAb molecules found as aggregates in cell culture samples was significantly altered by the protein-A chromatography step, with high molecular weight species (HMWS) decreasing by 20%. Samples that initially were enriched in monomeric mAb showed increases in HMWS following the protein-A chromatography step, whereas samples that were enriched in HMWS did not change significantly. These results indicate that protein-A chromatography can modify the aggregation state of monoclonal antibodies. The overall effect on the aggregate profile is dependent on the initial aggregate and monomer content of the sample.

2.2. Introduction

Aggregation of monoclonal antibodies that are used as therapeutic agents represents a safety concern for patients. Specifically, protein aggregates have been associated with immune responses [2, 3, 52]. For this reason, regulatory agencies expect that sponsors provide data to
show that aggregates in drug product can be controlled at acceptable levels. Due to the regulatory requirements and a sponsor’s interest in monitoring and controlling aggregation, extensive work has been done to understand where and how aggregates form in the monoclonal antibody manufacturing process [5, 53, 54]. For this reason, analytical methods are needed to quantify aggregate levels that can be applied throughout the protein production and purification processes.

Although robust methods are available to analyze mAb aggregation in highly purified solutions such as those characteristic of drug substance and drug product, analysis of mAb aggregation in more complex solutions such as unpurified mammalian cell culture broth is challenging. As a result, there are limited published studies that analyze the high molecular weight species (HWMS) content in such complex solutions [25, 31]. Two reasons exist for this lacuna. First is that the complexity of unpurified cell culture broth interferes with accurate size exclusion chromatography (SEC) analysis. Analysis of aggregate populations in cell culture broth typically has been performed by SEC only after an initial purification step is applied to reduce sample impurities such as background host cell proteins, nucleic acids, cell by-products and complex media components (i.e. amino acids, sugars, trace metals) [55], that can foul columns, reduce resolution and interfere with detection by UV absorption spectroscopy. Protein-A chromatography is typically used for this initial purification. Protein-A chromatography is a powerful purification step that utilizes an affinity ligand specific for antibodies [56]. The high specificity enables removal of up to 98% impurities from cell culture samples [57]. Typically, mAbs are first bound to the protein-A column under neutral pH conditions, washed to remove impurities and subsequently eluted at low pH (pH 3-5). Antibodies bind to the protein-A ligand through a combination of hydrophobic interactions, hydrogen bonds and salt bridges [58]. The strong interactions between the protein and ligand can be disruptive to the protein structure, and
especially under low pH conditions can result in protein aggregation [59-61]. We expect that the interactions and structural perturbations induced by the analytical protein-A chromatography process may alter the samples’ HMWS profile, as has been observed for other types of chromatographic separations processes [62, 63]. Limited work has been done to study the effect that protein-A elution has on the HMWS content of samples when used for sample preparation. Based on the existing literature, the low pH elution conditions can cause aggregation but the extent of impact has not been studied and reported.

A second reason for the data gap is that scientists have had limited motivation to quantify mAb aggregates in cell culture broth samples because mammalian cell culture historically yielded low mAb concentrations (<1 g/L) that were not thought to be associated with significant quantities of HMWS. However, in recent years, improvements in monoclonal antibody cell culture processes have resulted in dramatically higher mAb concentrations (2 to 20 g/L), and correspondingly higher levels of aggregates (10-20%) have been observed in SEC analyses conducted after pretreatment by protein-A chromatography [31]. This higher level of aggregates represents a significant amount of lost product and an added challenge for downstream purification processes.

To understand the impact of protein-A chromatography on mAb aggregate content in cell culture samples, we first developed an analytical SEC method that was able to directly resolve HMWS from the monomeric mAb in cell culture broth samples. Using this method, we measured HMWS levels in mammalian cell culture broth before and after purification by protein-A chromatography.

Two Chinese hamster ovary (CHO) cell lines were used in these studies. These cell lines (referred to hereafter as clone 59 and clone 40) are clonal cell lines formed by random
transfection of the parent cell line with the gene of interest. The clones were chosen because they both produce the same monoclonal antibody at similar, industrially relevant titers of 1-2 g/L (data not shown). Interestingly, both clones produce high levels of HMWS, but the levels produced by clone 40 are higher than those produced in cultures of clone 59.

In the first study, cell culture broth samples were purified using bench scale protein-A chromatography. The HMWS contents in the samples were measured by SEC before and after the protein-A chromatography step to determine if there was a shift in the aggregate profile during the protein-A chromatography step.

To explore whether protein-A chromatography might specifically alter the assembly state of HMWS or mAb monomers, a second study was performed using two solutions, one that contained purified and concentrated monoclonal antibody in monomeric form, and a second that contained purified and concentrated HMWS in addition to some monomeric mAb molecules. These solution components had been isolated from a clone 40 cell culture broth by preparative protein-A and size exclusion chromatography. Again, the purified and concentrated solutions were analyzed by SEC before and after a protein-A chromatography step to determine if protein-A chromatography shifted their aggregate content.

2.3. Materials and Methods

2.3.1. Cell line and media

Chinese hamster ovary (CHO) host cell lines were used to produce recombinant IgG mAbs. The host cells were co-transfected with split plasmids, one encoding the mAb heavy chain and the other encoding the light chain. A range of clones were isolated from different
transfections and different levels of amplification. Cell lines were cultured in spin tubes for the initial screening of clones.

Two clones (“clone 40” and “clone 59”) were selected in the initial screening process. These clones produce the same mAb at roughly equivalent, industrially relevant protein concentrations (3 g/L), yet analysis of their respective cell culture media by SEC following initial purification by protein-A chromatography showed that clone 40 produced five times higher levels of HMWS than did clone 59.

Cell culture scale-up steps were conducted in shake flasks (250 mL) and used a chemically-defined medium. Cultures were seeded to target 4x10^5 cells/mL for three day passages or 3x10^5 cells/mL for four day passages. Shake flasks were maintained at 36ºC, 6% CO₂ and were agitated on an orbital shaker at 150 RPM. For scale-up operations larger flasks (500 mL to 2 L) were used with increased working volumes (100 mL to 500 mL) and reduced rotation rates (130-140 RPM).

For the final production phase, the cell culture medium was switched to a modified chemically defined medium and shake flasks were seeded to target 5x10^5 cells/mL. Nutrient feeds were given on days 3, 6 and 8 at a fixed volumetric ratio (7% v/v). Base and glucose were fed as needed on the feed days to maintain pH between 6.9 - 7.0 and glucose concentrations between 8-12 g/L.

2.3.2. **Purified- concentrated monomer and HMWS solutions**

Monoclonal antibody monomer and HMWS fractions were isolated and purified from 2 L shake flask cell cultures of clone 40 cells after 10 days of cell culture production. On day 10, the
cells were removed by centrifugation at 500g for 5 minutes. The cell-free cell culture broth was
frozen for future processing.

Isolation of monomeric and HMWS fractions of the mAb was carried out using
preparative protein-A, size-exclusion chromatography and spin-filter concentration as detailed in
later sections. The protein concentrations for the final solutions were 0.3-0.9 g/L for the HMWS
solution and 15-16 g/L for the monomer solution. SEC analysis of the purified and concentrated
HMWS samples showed that they were composed of 80-90% HMWS with the balance as
monomeric mAb.

2.3.3.  **Preparative protein-A chromatography**

Protein-A chromatography used GE Health Care Life Sciences MabSelect SuRe resin
(Pittsburgh, PA) and was controlled using a GE Akta Explorer utilizing associated Unicorn
software. The resin was packed in a 2.1 cm diameter column with a 16.6 cm bed height. The
packed column was equilibrated with 0.025 M Tris, 0.1 M NaCl, pH 7.4 buffer and then loaded
with centrifuged cell culture broth to 20-35 g protein/L packed column volume. After washing
the column to elute loosely bound species, the bound protein was eluted using 0.05 M acetic
acid, pH 3.6 and elution was monitored by ultraviolet absorbance at 280 nm. The elution peak
was collected from baseline to baseline absorbance.

2.3.4.  **Preparative size exclusion chromatography**

Size exclusion chromatography used GE Health Care Life Sciences (Pittsburgh, PA)
Superdex 200pg resin and was controlled using a GE Akta Explorer utilizing associated Unicorn
software. The resin was packed into two stacks of 67 cm in 2.6 cm diameter columns. The
packed columns were equilibrated using 100 mM NaCl, 20 mM Na citrate, pH 6.9 buffer. mAb
samples that had previously been purified with protein-A were loaded at less than 1.5% mL/mL packed column volume. The protein was eluted using the equilibration buffer and the elution was monitored by ultraviolet absorbance at 280 nm. The elution peak was fractionated from baseline to baseline. A monomer pool was made by combining the elution peak fractions that corresponded to the monomer peak. The same was done for the HWMS fractions in order to generate a pool of mAb that was enriched in HMWS.

2.3.5. Concentration of mAb solutions

Solutions composed of protein-A eluate, SEC-purified mAb monomer fractions and SEC-purified mAb HWMS fractions were concentrated using 15 mL Millipore Centriprep™ spin concentrators with a 30 kDa molecular weight cutoff. Concentrators were rinsed twice with 100 mM NaCl, 20 mM Na citrate, pH 6.9 buffer by centrifugation at 1500 g for 15 minutes. Pools were concentrated in 15 mL aliquots by centrifugation at 1500 g for 10-40 minutes depending on the target final protein concentration.

2.3.6. Analytical size exclusion chromatography

High molecular weight species were quantified by SEC using a Waters Acquity (Milford, MA) UPLC system with a Waters (Milford, MA) BEH200 1.7 µm (4.6 mm x 150 mm) column. The column was equilibrated with phosphate buffered saline at 0.3 mL/min. Samples were thawed and centrifuged at 13000 g for 15 minutes then loaded to 20 µg with a 100 µL maximum injection volume. Protein elution was monitored by absorbance at 280 nm. Peak integration was performed to quantify percent HMWS, monomer and low molecular weight species (LMWS). Based on in-line light scattering analysis (data not shown), the HMWS peak contained mAb dimers and multimeric mAb species and the LMWS contained fragments (e.g. light or heavy
chains) of the complete mAb. Samples were analyzed out of chronological order and assay controls (bovine serum albumin and reference standard mAbs) were tested at the beginning and end of the sample set to ensure there was no assay bias over the course of the sample analysis.

Molecular weights of species in the eluate were determined using an inline multi-angle laser light scattering detector (Wyatt Technology (Santa Barbara, CA) miniDAWN Tristar laser photometer) which was connected to the outlet of the SEC-UPLC system. Wyatt Technology (Santa Barbara, CA) Astra software was used to collect and process the light scattering signal as a function of time. A refractive index increment $\frac{dn}{dc}$ of 0.186 [64] was used in the calculation of solute molecular weights.

2.3.7. Determination of mAb concentrations

mAb concentration in the cell culture broth samples was determined using protein-A affinity chromatography. The method used a high performance liquid chromatography (HPLC) Agilent Technologies (Santa Clara, CA) 1100 system consisting of a quaternary gradient solvent delivery system, ultra-violet light detector, autosampler with cooling capability and an Applied Biosystems (Grand Island, NY) POROS A/20 protein-A column (4.6 mm x 50 mm) monitoring at 280 nm.

Prior to sample analysis the thawed samples were centrifuged at 13000g for 5 minutes to remove insoluble precipitates. The supernatant was then transferred to HPLC vials. The column was equilibrated at 3 mL/min using 20 mM tris, 150 mM NaCl (pH 7.0), the sample was loaded to 9.5 – 210 µg protein, washed using 20 mM tris, 150 mM NaCl (pH 7.0) then eluted with 0.22 M acetic acid, 150 mM NaCl (pH 2.6). A purified mAb standard was analyzed at various
dilutions to generate a standard curve. The standard curve was used for calculating the sample protein concentrations.

mAb concentrations in the purified samples were measured by UV absorption at 280 nm using a C Technologies (Bridgewater, NJ) SoloVPE instrument.

2.3.8. Protein-A chromatography experiments

Purified-concentrated mAb monomer and mAb HMWS solutions were thawed and centrifuged at 2000g for 10 mins prior to loading. Protein-A chromatography used GE Health Care Life Sciences (Pittsburgh, PA) MabSelect SuRe resin and was controlled using a GE Akta Explorer utilizing associated Unicorn software. The resin was packed in a 1.1 cm diameter column with a 25 cm bed height. The packed column was equilibrated with 0.025 M Tris, 0.1 M NaCl, pH 7.4 buffer then loaded with concentrated aggregate to about 3 g protein/L packed column volume, representing about 10% of the loading for cell culture broth. After washing the column to elute loosely-bound species, protein was eluted using 0.05 M acetic acid, pH 3.6 and elution was monitored by ultraviolet absorbance at 280 nm. The elution peak was collected from baseline to baseline absorbance. Triplicate samples were analyzed for each sample treatment.

2.4. Results

In the production of therapeutic mAbs, aggregate levels typically are not measured directly in cell culture broth, but instead are measured (usually by SEC analysis) after first purifying cell culture samples using protein-A chromatography to remove cell broth constituents that might interfere with SEC analysis. In this study, we first developed a direct analytical SEC method that could resolve HWMS from monomer and cell broth constituents, allowing aggregates of a monoclonal antibody to be analyzed within cell culture broth without requiring
pretreatment by protein-A chromatography. Using a conventional UPLC size exclusion column, various buffers and running conditions were tested. The column resin and size were selected to have optimal resolution for >150 kDa proteins based on vendor literature. To reduce resin fouling a cleaning sample was run after every ten cell culture samples (0.5 M sodium sulfate). At the end of the sample set the column was cleaned using a sodium sulfate running buffer at 0.1 mL/min for 240 minutes then stored in 20% methanol (0.1 mL/min for 80 minutes).

Figure 2 shows the chromatogram associated with the analysis of cell culture broth samples using SEC. The HMWS peak has a retention volume of about 0.4CV and the monomer peak has a retention volume of about 0.5CV. The later peaks are associated with the low molecular weight cell culture broth constituents.

![SEC Chromatogram](image)

Figure 2. SEC chromatogram for Clone 40 cell culture broth samples (n=4) following freeze-thaw and centrifugation. HMWS peak has a retention time of 3 minutes. Monomer peak has a retention time of ca. 4 minutes. Peaks later than 5 minutes are small molecule constituents of the cell culture spent media.
Additional characterization was performed using light scattering and protein-A chromatography to confirm the identity of the monomer and HMWS peaks in the SEC chromatogram (Figure 3). As they eluted from the UPLC SEC, fractions were collected that contained the HMWS and monomer peaks. A light scattering detector was used to monitor the UPLC SEC eluate. Figure 3 shows that the molecular weight estimates align with expectations for the mAb monomer and HMWS. The dimer peak is not homogeneous based on the curvature of the light scattering data but the median molecular weight does align with the expected dimer molecular weight confirming the predominate species is dimer and not a larger oligomers. Structures presented in the figure are to illustrate the predominate eluting mAb species.

Figure 3. Chromatogram shows the light scattering intensity (red) and UV absorbance at 280 nm (dotted green). The HMWS peak elutes at 3.75 minutes with a molecular weight about twice the monomer. Monomer elutes at 4.25 minutes with a molecular weight aligning with the theoretical weight of 150 kDa. LMWS were also detected with a molecular weight representing about half a monomer. Example monoclonal antibody structures are shown to highlight the predominate species in the peak.
Samples of the collected HMWS and monomer fractions were analyzed by injecting them on a protein-A chromatography column, where strong binding to the resin indicated that the fractions contained mAbs. The protein-A chromatograms are shown in Figure 4. The fractions were not 100% pure HMWS or monomeric mAb, and both species are apparent in the protein-A chromatograms. The HMWS elute slowly over the course of 4CV.

Figure 4. Chromatograms of UV absorbance at 280 nm from analytical protein-A chromatography (A) HMWS enriched sample (B) monomer enriched sample. Monomer elutes at 2 minutes with the larger HMWS species eluting until 4.5 minutes.

Study 1 investigated whether the measured levels of HMWS changed after pretreatment of cell culture broth by protein-A chromatography. For samples from clone 59 cell cultures, the protein-A chromatography step removed all detectable HMWS (Table 1).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Clone 59 HMWS (%)</th>
<th>Clone 40 HMWS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Culture Production Flask After 10 Days</td>
<td>21 ± 1</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>Protein-A Chromatography Eluate</td>
<td>0</td>
<td>12 ± 1</td>
</tr>
</tbody>
</table>

Table 1. HMWS analysis by SEC for cell culture broth samples taken after ten days of cell growth and the corresponding HMWS results following protein-A chromatography of cell broth samples for each clone.

HMWS were reduced by approximately 20% (but not completely removed) following protein-A chromatography of samples from clone 40 cultures. These results suggest that HMWS is either being removed by the chromatography step or that HMWS is breaking down into monomer. The protein-A chromatography step yields for this study were calculated based on the grams of monomer loaded onto the protein-A column and the UV protein concentration and pool volume of the protein-A eluate (measure of total protein). These step yields were greater than 90% suggesting that the HMWS were not breaking down to form monomer, if so yields great than 100% would be expected, but were lost due to non-specific or weak binding to the affinity resin. The differences in HMWS purification across protein-A chromatography for the two clones suggests that the impact of the protein-A step on the HMWS content is protein-specific and that not all aggregates have the same binding affinity to the protein-A resin.

A second study was performed to understand how the HMWS content in samples that were enriched in mAb monomer or HMWS might change as a result of the protein-A chromatography step. Samples enriched in monomer and HMWS were generated by purification and concentration of clone 40 cell culture broth. The two samples (purified-concentrated monomeric mAb and mAb HMWS) were then loaded onto the protein-A column and the elute pool was analysed by SEC. The results from the experiments are shown in Figure 5.
Figure 5. SEC results for samples enriched in clone 40 monomer or HMWS pre and post protein-A chromatography purification. Data are shown for three species identified by SEC as LMWS, monomer and HMWS.

The monomer enriched sample initially contained 99% monomer (“Pre ProA”). Analysis of the sample following protein-A chromatography showed that the fraction of the sample composed of HMWS increased to 15 to 40% (“Post ProA”), indicating that the protein-A chromatography step generated HMWS. The reason for this could be due to protein structural perturbations during the binding or low pH elution steps that cause the protein to aggregate. These results indicate that if a protein-A chromatography step is used to prepare monomer-containing samples, the SEC results would not be representative of the original sample. The
sample that was enriched in HMWS showed no change in monomer and only small variations in the low molecular weight species (LMWS) across the protein-A chromatography column. This result may reflect a fairly high stability of HMWS during the protein-A chromatography step, or perhaps a dynamic disassembly-reassembly process for HMWS that yielded no net change in assembly state distribution.

2.5. Discussion

Prior to starting investigations work was done to determine operating conditions for an analytical SEC method that could be used to resolve HMWS in cell culture broth samples. Due to the complex nature of the cell culture broth samples, extensive resin cleaning was necessary to ensure consistent sample analysis over the course of a sample set. A cleaning step was performed after every ten cell culture samples to reduce resin fouling. Additionally, an aggressive cleaning method was used at the end of a sample set to remove any residual cell culture constituents. The analytical SEC method was shown to consistently resolve monomer and HMWS in cell culture samples.

Study 1 investigated the change in HMWS content of cell culture broth samples following protein-A purification. Purification of cell culture broth samples using protein-A chromatography had a significant impact on the HMWS content of samples (Table 1). This was observed for samples from both clone 59 and 40 cell culture broths. Clone 59 cell culture broth sample had a larger decrease in HMWS content across protein-A purification compared to the clone 40 cell culture broth sample. The difference in protein-A chromatography HMWS clearance between the cell culture broth samples from clone 59 and clone 40 suggests that the impact of the protein-A step on the HMWS content is related to differences in the HMWS
characteristics (for example, aggregate size distribution, proportion of covalent/non-covalent aggregates, or ratio of monomer to monomer fragments) that would impact the HMWS affinity for protein-A resin. Shukla et al. documented that protein-A eluate pools for 14 monoclonal antibodies had various levels of soluble aggregate as measured by SEC [61], confirming that the modulation of HMWS across protein-A chromatography is not consistent.

The second study analyzed samples that were enriched in either HMWS or monomer purified from clone 40 cell culture broth. The monomer enriched sample had a 30% increase in HMWS across protein-A purification while the HMWS enriched sample did not change significantly. The contrast between the study 1 and 2 results suggests that the load sample type (cell culture broth vs. purified-concentrated monomer vs. purified-concentrated HMWS) plays a role in the resulting protein-A pool HMWS content. The initial HMWS concentrations in the various samples were significantly different, ranging from 0 to 80% for enriched samples and 20 to 30% for the cell culture broth samples. This broad range was based on the extent of purification performed and this therefore created heterogeneous aggregate populations for each sample that drove changes in aggregation kinetics or resin binding potential. In study 1, cell culture samples decreased in HMWS content by 20%. In study 2, purified-concentrated monomer samples increased in HMWS by 15 to 40%. A potential explanation for these results is that, under column binding and/or elution conditions, disassembly/reassembly kinetic processes for HMWS may cause the monomer/HMWS assembly state distributions to approach an equilibrium. Shukla et al. showed that aggregation in protein-A pools for an Fc fusion protein followed first order reaction kinetics and that the protein interaction with the resin increased the protein aggregation propensity. Additionally, a similar observation was made by Gabrielson et al. in a study of the effect of processing by SEC on distributions of HMWS for mAbs. Their
results showed that HMWS distributions in the SEC eluate were dependent on the starting sample aggregate contents and that there were shifts in the distribution of protein assembly state populations between monomer, dimer and trimer during purification.

2.6. Conclusions

The results presented from these studies demonstrate the extent to which protein-A chromatography can change the HMWS distribution for a mAb in cell culture samples and the dependence it has on the initial HMWS content of the sample.

The first study showed that mAb HMWS percentage in samples of cell broth decreased after processing by protein-A chromatography. This is likely due to removal of mAb HMWS that were weakly-bound to the affinity resin. The second study showed that processing of monomer enriched samples by protein-A chromatography could increase the mAb HMWS content due to additional aggregation of monomer. We speculate that the strong interactions between the mAb and protein-A ligand are disruptive to the mAb structure, and combined with low pH conditions perturb the protein stability fostering aggregation.

Taken together, these studies confirm and quantify the HMWS shift that results from purifying cell culture samples by protein-A chromatography prior to analysis by SEC. As shown by methods developed for this research, direct analysis of cell culture samples for aggregate content is possible, and recommended to yield more representative results, enabling better process development decisions.
Chapter 3: Aggregation of a Monoclonal Antibody in Mammalian Cell Culture Media

3.1. Abstract

Protein aggregates represent a safety, immunological and stability concern in therapeutic protein formulations. Aggregates may be formed at any stage during the manufacturing process, including during cell culture. The proteins that are produced in mammalian cell culture typically are incubated in the cell culture medium at relatively high temperatures (e.g. 36 °C) for prolonged periods of time (1-2 weeks). To examine potential mechanisms by which this incubation might generate protein aggregates, we examined the stability of a model monoclonal antibody (mAb) under cell culture conditions, but in the absence of cells. The mAb was produced in two different cell lines. Cells were removed from cell culture media and the resulting supernatants were incubated at 36 °C for up to ten days, during which time aggregation of the mAb was monitored by size exclusion chromatography. The mAb was stable when added to fresh cell culture media but formed aggregates when added to spent media (without cells) that contained proteins and other cellular by-products. After ten days of incubation in the cell-free spent medium, 10-35 % of the mAb formed high molecular weight species. One of the cell lines (clone 40) formed HMWS at a level that was three times higher than that in the corresponding cell-free spent medium from the other cell line (clone 59). Thus, although the aggregation of the mAb occurred extracellularly, the cell line influenced the propensity of the mAb to form aggregates. Background proteins in the clonal spent media likely contributed to the propensity for the mAb to aggregate.
3.2. Introduction

Monoclonal antibodies (mAb) represent an important class of therapeutic agents used to treat a variety of human diseases including cancer, inflammation, metabolic disorders and bone disease [65]. As with any protein, monoclonal antibodies can aggregate in solution. In particular, mAbs frequently aggregate to form high molecular weight species (HMWS), where HMWS are a subgroup of aggregates comprised of dimers and larger oligomers. As a working definition, HMWS are mAb aggregates with size and solubility characteristics that allow them to be analyzed by size exclusion chromatography. The formation of these HMWS may have significant impact on the manufacturing process and can raise potential safety concerns [3, 66].

Recent developments in mAb production have led to mammalian cell culture processes that can generate mAbs at concentrations reaching tens of grams per liter. Unfortunately, these remarkable protein concentrations frequently are associated with the formation of high concentrations of HMWS in the cell culture. This correlation is not surprising based on the extensive work showing increased aggregation rates at higher protein concentrations [4-8]. Because these HMWS are discarded when the monomeric mAbs are purified downstream of their production in cell culture, they represent lost product. In addition, HMWS may increase the complexity of downstream processing (e.g., choice of chromatography resin and number of purification steps), and, if they are not completely removed during purification, may impact the safety and stability of the drug product [3]. Due to the impact of mAb aggregation on the manufacturing process and its potential to affect product safety and stability, it is desirable to understand (and ultimately inhibit) aggregate formation mechanisms in the cell culture process.

HMWS formation during cell culture has received only limited attention for two primary reasons. First, the very high mAb concentrations that can now be realized, and the concomitantly
high levels of aggregation observed during cell culture are relatively recent phenomena. Second, analytical techniques for measuring aggregate concentrations in cell culture samples have been limited due to the solution complexity. In addition to monomeric mAbs and HMWS, the complicated mixtures formed during cell culture contain various unconsumed cell culture medium components, metabolites, other proteins and cell debris. Most available techniques to analyze HMWS require that these complex components be removed prior to analysis of HMWS, which could confound results [67]. Typically, cell culture samples, which contain mAb that is 70-90% monomeric, are centrifuged to remove cell debris and then purified across a protein-A affinity chromatography column before being analyzed by size exclusion chromatography (SEC) [54]. This is in contrast to analyses of highly purified (> 90% purity) drug product samples that typically require no additional purification prior to analysis.

Despite the difficulties of studying the mechanisms of protein aggregation in cell culture media, early work suggests that protein aggregation during cell culture occurs extracellularly in the cell culture medium [25, 53, 54]. Factors that could contribute to extracellular aggregation include solution pH, osmolality or ionic strength [5-7, 15, 21-23], media components or additives [5-7, 15, 24-29], solution temperature [5, 7, 15, 30, 31], protein concentration [4-8] and solution redox potential [20, 25].

In one study, Cromwell showed that mAb aggregation (as measured by size exclusion chromatography following initial purification by protein-A chromatography) occurred at a constant rate throughout the time-course of the cell culture process, and continued at that rate in the absence of cells, suggesting that HMWS were not being directly created in and secreted from cells. [54]. Furthermore, the protein concentration-independent rate of production of HMWS
suggests that high mAb concentrations alone cannot explain the high concentrations of HMWS seen in current cell culture processes.

Recent work by Jing using Chinese hamster ovary (CHO) cells that produced recombinant IgG fusion proteins showed that various cell culture conditions, including temperature, dissolved oxygen, pH, cystine concentration and bivalent copper ion concentration impacted aggregation [25]. Specific mechanisms by which these cell culture conditions affected aggregation were not provided.

Additionally, Dengle performed stability studies using 11 monoclonal antibodies [68]. In these studies the antibodies were held under various shaking and stirring conditions at 36.5°C in two cell culture medium formulations in the absence of cells [68]. Aggregation was monitored over a six-day incubation period and measured by size exclusion chromatography after an initial filtration through a 0.22 µm filter. Eight of 11 monoclonal antibodies aggregated in the fermentation medium. This confirms that under simulated cell culture conditions of stirring, elevated temperature and media components monoclonal antibodies may aggregate even in the absence of cells and that aggregation propensity can be mAb specific.

Ultimately, there may be multiple extracellular factors that contribute to an overall increase in aggregate levels. In this study, a step-wise approach was taken to narrow down potential contributing factors for cell culture protein aggregation. The step-wise approach started with examination of mAb stability in simple solutions (buffer and fresh cell culture media) then progressed to cell-free cell culture spent media containing cell by-products, nutrients and proteins. Three cell lines were used to examine mAb stability in cell-free cell culture media. The first cell line was a null cell line that did not express the mAb protein, but was otherwise identical to the mAb-producing cell lines. Spent cell culture medium obtained after removing
cells from the null cell line therefore contained cell by-products, nutrients and metabolites, but no mAb molecules or mAb fragments. Two mAb-producing clonal cell lines were also used. These clones each expressed the same mAb at roughly identical expression levels but with different HMWS contents. Culture media obtained after the cells were removed from cultures of these clonal cell lines therefore contained cell by-products, nutrients, metabolites, mAb and mAb fragments. Due to differences in the clonal cell line expression and cell growth, the concentrations of the cell-free medium components (by-products, nutrients, metabolites and background proteins) were also different.

3.3. Materials and Methods

3.3.1. Cell lines and cell culture media

The cell lines used were Chinese hamster ovary (CHO) cell lines. The first CHO cell line was designed to not express the mAb of interest. This cell line is referred to as a null cell line as it lacks the plasmids that code for the mAb. The purpose of using a null cell line was to create samples of cell culture media that contained appropriate background host cell proteins, but no mAb, mAb HMWS or mAb fragments. In turn, purified mAb could be spiked into this cell culture medium in order to study the stability of the mAb in the absence of mAb fragments or HMWS.

Clonal CHO cell lines producing recombinant IgG monoclonal antibodies were also used. A range of clones were isolated from different transfections and different levels of amplification, and stored in vials in the frozen state.

Two clones (hereafter referred to as clones 40 and 59) were selected that produce the same mAb at similar, industrially-relevant protein concentrations (1-2 g/L) and yet also produce
significantly different levels of soluble, predominately dimer sized, HMWS, based on analysis by 2D SEC (data not shown). The stability and product characteristics of the mAb monomer were assessed and found to be representative of a clinically viable mAb.

Developmental cell banks were created by thawing vials containing each of the two clones and expanding the cultures into 250 mL shake flasks using a chemically-defined medium. After 3-4 passages cells were harvested by centrifugation and re-suspended into chemically defined medium with 10% by volume DMSO. After the contents of the vials were frozen in liquid nitrogen, a single vial from each cell bank was thawed and tested to confirm viability and cell density. Recovery of viability of the clones was greater than 90%, with acceptable viable cell density based on the targeted fill density.

Cell culture scale-up steps used a chemically-defined medium. For the production phase, the medium was switched to a modified chemically-defined medium that was selected for optimal cell growth and protein production.

3.3.2. **Shake flask experiments**

Experiments were carried out in 250 mL shake flasks with a starting volume of 50 mL. Cultures were seeded to target 4x10^5 cells/mL for three-day passages or 3x10^5 cells/mL for four-day passages. Cultures were maintained at 36° C, 6% CO₂ and rotated at 150 RPM. For scale-up operations, larger flasks (500 mL, 1 L and 2 L) were used with increased working volumes and reduced rotation rates.

For the production phase, nutrients were added on days 3, 6 and 8 at a fixed volumetric ratio. Base and glucose were fed as needed on the feed days to maintain pH between 6.9 - 7.0 and glucose concentrations between 8-12 g/L. The cultures were sampled on feed days to test for
metabolite concentrations, pH, pCO₂, pO₂, cell viability, cell density, mAb titer and aggregate content. Metabolite concentrations were measured offline using a Bioprofile Analyzer 100+ (Nova Biomedical Corporation, Waltham, MA). pH, pCO₂ and pO₂ were measured on a Rapidlab 860 blood gas analyzer (Bayer Healthcare, East Walpole, MA). Cell viability and density were measured offline using a Cedex 2.1 automated cell counter (Innovatis AG, Bielefeld, Germany). Samples were frozen at -70°C for future analysis. All measurements were carried out in duplicate unless otherwise noted.

3.3.3. Preparative protein-A chromatography

Protein-A chromatography used GE Health Care Life Sciences MabSelect SuRe resin and was controlled using a GE Akta Explorer utilizing associated Unicorn software. The resin was packed in a 2.1 cm diameter column with a 16.6 cm bed height. The packed column was equilibrated with 0.025 M Tris, 0.1 M NaCl, pH 7.4 buffer then loaded with centrifuged cell culture broth to 20-35 g/L packed column volume. After washing the column to elute non-tightly bound species, protein was eluted using 0.05 M acetic acid, pH 3.6 and elution was monitored by ultraviolet absorbance at 280 nm. The elution peak was collected from baseline to baseline absorbance.

3.3.4. Preparative size exclusion chromatography

Size exclusion chromatography used GE Health Care Life Sciences Superdex 200pg resin and was controlled using a GE Akta Explorer utilizing associated Unicorn software. The resin was packed into two stacks of 67 cm in 2.6 cm diameter columns. The packed columns were equilibrated using 100 mM NaCl, 20 mM Na citrate, pH 6.9 buffer. Samples containing mAb and mAb HMWS that had been pre-purified using protein-A chromatography were loaded at less
than 1.5% mL/mL packed column volume. The protein was eluted using the equilibration buffer and the elution was monitored by ultraviolet absorbance at 280 nm. The elution peak was fractionated from baseline to baseline, and collected fractions were pooled to create separate HMWS- and monomeric mAb-containing pools.

3.3.5. **Centriprep concentration**

Pooled samples were concentrated using 15 mL Millipore centriprep spin concentrators with a 30kD molecular weight cut off. Prior to their use, concentrators were rinsed twice with 100 mM NaCl, 20 mM Na citrate, pH 6.9 buffer by centrifugation at 1500 g for 15 minutes. Pooled samples were concentrated in 15 mL aliquots by centrifugation at 1500 g for 10-40 minutes depending on the target final protein concentration.

3.3.6. **Monomer and aggregate spike generation**

Some experiments examined the effect of “spiking” in concentrated monomeric mAb or aggregated mAb into various test solutions. Pools of purified, concentrated mAb monomer or purified, concentrated mAb HMWS were isolated and purified from shake flask cell culture broth for each clone. To generate sufficient material, multiple 2 L flasks were cultured and harvested by centrifugation. The cell-free cell culture broth was stored frozen prior to being thawed and then purified using preparative protein-A, size-exclusion chromatography and centriprep concentration. The protein concentrations for solutions used for spiking studies were 0.3-0.9 g/L for the HMWS pools and 15-16 g/L for the monomer pools. The mAb content of the HMWS spike material was composed of 80-90% HMWS by SEC, with the balance as mAb monomer.
3.3.7. **Drug substance spike generation**

In some studies, drug substance material composed of the same mAb was spiked into various solutions to test the effect of solution conditions on mAb stability. mAb drug substance material was obtained from a large-scale purification run. The product purity profile for drug substance antibodies was similar to those produced in the cell lines (clone 40 and clone 59) used for bench-scale studies. The final drug substance formulation buffer contained 10 mM sodium acetate, 9% sucrose and 0.004% Tween-20® at pH 5.2.

3.3.8. **Protein concentration determination**

Protein concentrations were determined using analytical scale protein-A affinity chromatography. The method used a high performance liquid chromatography (HPLC) Agilent 1100 system consisting of a quaternary gradient solvent delivery system, ultra-violet light detector monitoring at 280 nm, autosampler with cooling capability and an Applied Biosystems POROS A/20 protein-A column (4.6 mm x 50 mm).

For sample analysis the thawed samples were centrifuged at 13000 g for 5 minutes. The supernatant was then transferred to HPLC vials. The column was equilibrated at 3 mL/min using 20 mM tris, 150 mM NaCl (pH 7.0), the sample was loaded to 9.5 – 210 µg protein, washed using 20 mM tris, 150 mM NaCl (pH 7.0) then eluted with 0.22 M acetic acid, 150 mM NaCl (pH 2.6). A purified protein standard was analyzed at various dilutions to generate a standard curve. The standard curve was used for calculating the sample protein concentrations.
3.3.9. **Analytical size exclusion chromatography for HMWS determination**

HWMS were quantified using size exclusion chromatography. The method used a Waters Acquity UPLC system with a BEH200 1.7 µm (4.6 mm x 150 mm) column. The column was equilibrated with 1x phosphate buffered saline at 0.3 mL/min. Samples were thawed and centrifuged at 13000 g for 15 minutes then loaded to 20 µg with a 100 µL maximum injection volume. Protein elution was monitored by absorbance at 280 nm. Peak integration was performed to quantify percent mAb HMWS, mAb monomer and LMWS. Based on in-line light scattering detector data, the HMWS peak contained dimeric and multimeric species and the LMWS contained fragments of the mAb monomer with an average molecular weight of 85 kDa. Bovine serum albumin was run as an assay control with the expectation that the relative standard deviation for five injections be less than 5% for the HMWS and main peak areas. Samples were analyzed out of chronological order and assay controls (BSA and reference standard) were run at the beginning and end of the sample set to ensure there was no assay bias over the course of the sample analysis. Error bars were calculated as one standard deviation. For experiments using drug substance, only a single flask was used and thus no error bars are reported.

3.3.10. **Monoclonal antibody stability studies**

To determine if the monoclonal antibody aggregated in the absence of cells, mAb or mAb HMWS were spiked into one of four solutions, incubated at 36°C and monitored for levels of HMWS. The four test solutions contained either phosphate buffered saline (buffer), fresh media, null cell line spent medium or clonal cell line spent medium. Spent media (also termed cell-free cell culture media) were generated by culturing cells for three days then centrifuging the cell culture broth at 500 g for 5 minutes to remove the cells. The supernatant was aseptically
transferred to sterile 250 mL shake flasks with an initial working volume of 50 mL. Previously isolated purified-concentrated monomer and HMWS pools (spikes) were used to increase the monomer or aggregate concentration in the various solutions. The spiking solutions (containing purified-concentrated monomer, purified-concentrated aggregate or drug substance) were added using a 0.22 µm syringe filter and injected directly into the shake flask in a laminar flow hood. The conditions studied are described in Table 2. Table 2 details the stability solution tested, the material that was spiked into the stability solution and the target concentration for calculating the spike volume. Flasks were placed in an incubator at 36°C with 6% CO₂ with rotation at 150 RPM. Samples were taken on days 0, 3, 6, 8 and 10 of the incubation period and stored frozen at -70°C.
Table 2. Experimental conditions for monoclonal antibody stability studies were defined by: the background solution (spent medium from clone 59 or 40 day 3 cell culture, buffer or fresh media), the concentrated spike solution (purified-concentrated aggregate from clone 59 or 40 cell cultures, purified-concentrated monomer from clone 59 or 40 cell cultures, drug substance) and the target concentration for calculating the spike volume into the stability flask.

3.4. Results

3.4.1. Assessment of protein aggregation in the presence of cells

Clone 40 and clone 59 cell lines were cultured and sampled during the production phase to understand the baseline aggregation levels with cells present. Figure 6 shows the change in HMWS levels during the cell culture production phase with cells present. The two clones behaved differently, with the cell culture medium from clone 40 cultures showing a relatively linear increase in HMWS levels, whereas HMWS levels in clone 59 cultures increased slightly until day 8 then decreased until the end of production.
Figure 6: Time-course mAb HMWS formation during cell culture for clone 59 (solid line) and clone 40 (dashed line) cell lines. Cell cultures were sampled on days 3, 6, 8 and 10. Samples were centrifuged to remove cells, frozen at -70°C, thawed and analyzed by SEC.

3.4.2. **Assessment of protein aggregation in the absence of cells**

Fresh cell culture medium or buffer were spiked with drug substance or monomeric mAb purified from clonal cell line cultures and incubated at 36 °C for 10 days in order to determine the stability of the mAb in these solutions. Samples spiked with monomeric mAb or with mAb drug substance initially had low levels of HMWS. As shown in Figures 7 and 8, slight decreases in HMWS contents were observed over the ten-day period of incubation. This suggests that HMWS may disaggregate over time, and also suggests that the monomeric mAb is stable in fresh media and buffer.
Figure 7: Buffer (1x PBS) was spiked with clone 59 mAb monomer (solid), clone 40 mAb monomer (dashed line) or drug substance (dotted line) to target 1.5 mg/mL and incubated at 36°C with shaking. HMWS formation was monitored over ten days by SEC. No increase in HMWS could be detected as a result of mAb incubation in buffer or media with elevated temperature and agitation.

Figure 8. Fresh, cell-free culture medium was spiked with clone 59 mAb monomer (solid line), clone 40 mAb monomer (dashed line) or drug substance (dotted line) to target 1.5 mg/mL and incubated at 36°C with shaking. HMWS formation was monitored over ten days by SEC. No significant increase in HMWS could be detected as a result of mAb incubation in fresh cell culture medium with elevated temperature and agitation.
To determine if cell by-products and metabolites contribute to the propensity of the mAb to aggregate, a null cell line spent medium was used as a test solution. The null cell line does not produce the mAb, resulting in a spent medium solution that is lacking background mAb monomer, LMWS and aggregates, but that contains other cell by-products and metabolites. During the cell culture production phase spent media was harvested on day 3 by centrifugation to remove the cells. Monomeric mAbs purified from a previous clonal cell culture run were spiked into the spent media and monitored for protein aggregation using SEC. The spiked-in clonal mAb monomer appeared to be stable under cell culture conditions; no significant increases in the relative HMWS concentrations were observed (Figure 9).

![Graph](image)

Figure 9: Spent media from a null cell line was spiked with clone 59 monomer (solid line) or clone 40 monomer (dashed line) to target 1.5 mg/mL and incubated at 36°C with shaking. HMWS formation was monitored over ten days by SEC. With the exception of lacking mAb-associated proteins, these solution conditions are expected to mimic those found in the spent media from clonal cultures, but they induce little or no mAb aggregation.
Clonal cell culture spent media were used to assess protein stability in the absence of cells but in the presence of background mAb and mAb fragments. During the cell culture production phase spent medium was harvested on day 3 and centrifuged to remove cells. Aggregates and monomers previously purified from a clonal cell culture run were spiked into the spent media, incubated at 36°C and monitored for protein aggregation using SEC. Analysis of spent media spiked with mAb aggregates as well as un-spiked spent media showed an increase in percent HMWS over the 10 day incubation period (Figures 10A, B). Spent media that were spiked with mAb monomer or drug substance showed little increase in percent HMWS, in part due to the high concentration of monomer, although the absolute concentration of HMWS increased. The increasing absolute levels of HMWS showed that all conditions fostered some level of aggregation (Figures 10C, D).
Aggregation occurred in the clone 40 spent medium spiked with drug substance and in the clone 40 spent medium spiked with monomer, but to a lesser extent compared to aggregation observed un-spiked clone 40 spent medium or clone 40 spent medium spiked with concentrated
aggregate. This observation suggests that the monomer or drug substance population might be inhibiting aggregation. Clone 40 showed higher levels of aggregation compared to clone 59. This difference was previously observed during the selection and development of the clonal strains.

SEC analysis resolved three populations of mAb-related species based on size, specifically, HMWS, monomer and LMWS. Light scattering results indicated that the HMWS is primarily dimerized mAb (~300-400kDa) and the LMWS is primarily half of a mAb monomer comprised of one heavy chain and one light chain (~85kDa). To understand how the aggregates form during the stability studies, changes in the percent HMWS were compared to changes in the percent LMWS and monomer. Results showed that increases in HMWS were balanced by decreases in monomeric mAb (Figure 11). This indicates that monomers are contributing to HMWS formation and half mAb fragments are not. Figure 11 represents the un-spiked conditions for each clone; similar responses were observed in spiked conditions.

Figure 11: Clone 40 spent media (A) with no spiked monomer or aggregate was incubated at 36°C with shaking. Clone 59 spent media (B) with no spiked monomer or aggregate was incubated at 36°C with shaking. Aggregation was monitored by SEC over ten days. Results show that as HMWS (solid circle) increases there is a decrease in monomer (open circle) and no change LMWS (solid triangle).
To investigate the protein stability relative to the spent medium, mAb aggregates produced in clone 40 cultures were spiked into spent media from clone 59 cultures, and vice-versa. Aggregation levels in these “cross-spiked” samples were compared against levels seen in un-spiked spent media or spent clonal media spiked with mAb aggregates produced by the same clone. Previously purified-concentrated HWMS were added to the spent medium to target 0.03 mg spike protein per ml spent medium. The volume of spike solution added was calculated based on the total protein concentration of the spike solution measured by absorbance at 280 nm. The spent media were harvested after three days of production resulting in a background mAb protein concentration of approximately 0.1 mg/mL as measured by analytical protein-A chromatography. Using this spiking strategy resulted in HMWS percentages that were 2-3 times higher than in the un-spiked conditions. Aggregation trends within solutions of each clonal spent medium were similar for each condition regardless of the nature of spike material (Figure 12).
Clone 40 spent medium resulted in higher levels of final HMWS compared to final HMWS levels in clone 59 spent medium conditions indicating that clone 40 spent medium promoted higher levels of aggregation than did spent medium from clone 59 cultures. This was also observed in the cross-spike condition as clone 59 aggregate conditions had higher final HMWS in clone 40 spent medium compared to clone 59 spent medium. Due to the high levels of background protein (80% of the total protein concentration), it was not possible to discern whether the observed aggregation was due to the spiked mAb proteins or to the mAb proteins present in the spent media prior to spiking.
3.5. Discussion

Monitoring protein aggregation in cell culture is challenging due to significant background interference from cells, background proteins, and cell culture medium components, but the importance of such analyses are increasing as the levels of aggregation observed in cell cultures increase concomitantly with protein concentrations. Because the protein-A chromatography steps that are commonly used to pre-treat cell culture samples prior to analysis of mAbs and mAb HMWS may alter the distributions of mAb assembly states (Russell and Randolph, 2015, submitted), it is important to minimize the extent of pre-treatment before SEC analysis of mAb HMWS. Despite the complicated composition of cell culture media, newer SEC systems can be used to analyze mAbs and mAb aggregates within cell culture-based samples with minimal sample preparation (e.g. protein-A purification). This advancement avoids extensive pre-treatment steps, and allows for a more direct assessment of protein aggregation in cell culture.

Direct SEC analysis of mAb and mAb HMWS in cell culture media showed that when the same mAb was produced in two different clonal cell lines, aggregation occurred to different extents. However, the aggregates were not produced directly by the cells of each clone, because aggregation continued even after cells were removed from the cell culture media.

Interestingly, after clone 40 cells were removed, the clone 40 spent cell culture medium produced roughly the same high level of aggregates whether the spent medium was un-spiked, or spiked with mAbs aggregates produced by clone 40 or with mAbs aggregates produced by clone 59 (Figure 12A). Likewise, after clone 59 cells were removed, the clone 59 spent cell culture medium produced roughly the same relatively low level of aggregates independent of whether the spent medium was un-spiked, or spiked with mAbs aggregates produced by clone 40 or by
clone 59. In addition, aggregation was not observed in the absence of cells when mAbs were added to the starting cell culture medium, or in buffer solutions (Figures 7 and 8). Thus we infer that aggregation of the mAb was not due to inherent protein instability under cell culture conditions (i.e. temperature, agitation and media chemical components). Rather, aggregation is due to factors related to the extracellular composition which include metabolites and background proteins. Presumably, differences in aggregation rates for mAb in cell-free spent media from each of the clones are related to clonally-generated differences in the composition and/or quantities of these by-products or background proteins.

What is the likely identity of the cellular by-products or background proteins that promoted mAb aggregation? Some insight may be gained from the experiments with spent medium from the null cell line cell culture. This spent medium lacks the mAb but contains representative levels of typical cell culture medium chemical components, nutrients, metabolites, and host cell proteins. When monomeric mAb was spiked into spent media obtained after removing the null cells, little or no aggregation was observed (Figure 9). Thus, it appears that the medium chemical components, nutrients, metabolites and host cell proteins alone do not cause the observed protein aggregation in cell culture, and we surmise that by-products of mAb production itself such as the incompletely assembled mAb molecules and mAb fragments present in the cell culture media of the mAb-producing clones are contributing to the observed aggregation.

3.6. Conclusions

The results support the hypothesis that proteins can aggregate extracellularly. The mAb is stable in buffer, fresh medium and null cell line spent medium. This eliminates several factors
that potentially could cause protein aggregation, including: normal cell culture conditions (36°C with shaking), inherent protein instability, media components/nutrients, cell metabolites and host cell proteins. Under the same temperature and shaking conditions, mAb aggregation occurs in spent media from the two clonal cultures, even in the absence of cells. This aggregation was independent of which clone produced the mAb, but depended strongly on the clone that was used to produce the spent media. This observation strongly supports the hypothesis that mAb-associated by-products (e.g. HMWS or LMWS) contribute to the aggregation propensity.
Chapter 4: Monoclonal antibody aggregation in cell culture is mediated through extracellular light chains

4.1. Abstract

Production of monoclonal antibodies (mAbs) at high titers in commercial mammalian cell culture reactors is often associated with concurrent production of protein aggregates. We hypothesized that proteins produced by the cells, and in particular excess mAb light chains, contributed substantially to this aggregation. To probe whether excess light chains were associated with aggregation, aggregates were isolated from the cell culture and analyzed to determine their composition of mAb monomers, low molecular weight species (LMWS), mAb light chains and mAb light chain dimers. Aggregates contained an excess of mAb light chains. Samples of mAb light chains were incubated under cell culture conditions in solutions that contained typical levels of mAb monomers, LMWS and mAb-associated high molecular weight species (HMWS). Analysis of the samples showed that HMWS levels increased and free light chain levels decreased as a function of incubation time. Lastly, to confirm that mAb light chains are factors in aggregation, spent medium from cell culture reactors was treated with protein-A resin to remove mAb, mAb HMWS, and free mAb light and heavy chains. Purified mAb monomers were then spiked into the protein-A treated spent medium and aggregation was monitored for ten days using size exclusion chromatography. In the absence of background mAb species, the spiked-in monomers were stable and did not form aggregates. The results from aggregate characterization and mAb stability studies are consistent with free light chain mediated protein aggregation.
4.2. Introduction

Proteins are used as therapeutic agents to treat diseases including cancer, inflammation, metabolic disorders, and bone disease [65]. As an expectation in regulatory approval of a protein therapeutic, the sponsor must demonstrate safe and acceptable levels of protein aggregates. Protein aggregates in drug product represent a safety concern as they have been associated with undesirable immune responses [2, 3, 52]. Based on the need to monitor and control aggregation in a biotechnology manufacturing process, extensive work has been done to understand influencing factors and aggregation pathways [5, 11, 54].

Monoclonal antibodies are a subclass of therapeutic proteins of increasing clinical importance [1]. The manufacturing process for producing and purifying mAbs is complex [69], with unit operations that require operating conditions that are not conducive to protein stability. As a result, mAbs may aggregate during their production [54]. In particular, the agitated mammalian cell culture bioreactors used to produce mAbs typically are operated at elevated temperatures for production cycles that may last for weeks. Aggregates formed during cell culture must be removed in downstream purification processes. An increased understanding of protein aggregation mechanisms under cell culture conditions would enable better control of aggregation and thereby improve the product safety profile, increase process yield, and reduce the need for extensive downstream clearance capability.

Protein aggregation in cell culture has been correlated with intracellular monoclonal antibody light chain (LC) and heavy chain (HC) levels [32-34, 36-39]. In particular, Ho et al. found that a high intracellular peptide ratio of light chains to heavy chains was associated with
the presence of light chain dimers and low levels of aggregates outside of the cell [39]. Conversely, they saw that when heavy chain peptide was in excess inside of the cell, LC:HC ratio of 0.3, there was an increase in aggregates and mAb fragments outside of the cell. Additionally, using two mAbs that express similar LC:HC ratios, they observed different levels of aggregation suggesting that the aggregation propensity is antibody or cell line dependent. Although aggregates were detected by analysis of the extracellular cell culture broth, the studies did not identify whether the aggregates were forming intracellularly or extracellularly.

Ishii et al. investigated mAb aggregation in cell culture using two clonal cell lines, referred to as cell lines A and B [34]. Cell line A was associated with having a lower aggregation propensity compared to cell line B as measured by extracellular HWMS using size exclusion chromatography. The study suggested that cell line B was under a cellular stress response due to accumulation of intracellular HC which resulted in secretion of aggregation prone misfolded mAb species. This was supported by higher levels of extracellular LMWS, lower levels of extracellular and intracellular LC and LC dimer, high levels of intracellular HC and up-regulation of BiP and PDI expression.

Lee et al. correlated mRNA expression levels for HC and LC to levels of aggregates in cell culture supernatant [36]. Analysis of 17 cell lines, each expressing the same antibody, showed a positive correlation between the mRNA HC:LC ratio and the aggregate levels measured in the cell culture supernatant, consistent with an interpretation that inadequate levels of intracellular LC led to higher extracellular aggregate levels. The proposed mechanism of aggregation was that when intracellular LC levels are insufficient, the HC is prone to misfolding and aggregation [33, 35]. Although the aggregates that Lee et al. detected were extracellular, it was not specified if they had been formed intracellularly or extracellularly.
A common theme in the previous work was the suggestion that intracellular LC limitations constrain the protein folding process, resulting in the production of mAb and mAb species that are more prone to aggregation. As HC is not readily secreted, extracellular aggregation would likely involve LMWS excluding HC species. Thus, we sought to explore whether LMWS, specifically LC and LC dimer, might play an important role in extracellular aggregation of mAbs under cell culture conditions using two Chinese hamster ovary cell lines expressing the same mAb. We first isolated and purified HMWS from mammalian cell culture broths and characterized their composition. Additionally, we examined temporal changes in LC/LC dimer and HMWS concentrations during incubation under cell culture conditions to determine if production of HMWS correlated with extracellular LC/LC dimer concentrations. Lastly, to test the theory that background mAb species were involved in aggregation, we treated cell-free cell culture (spent medium) to remove background mAb species and monitored the stability of spiked in, purified mAb monomer.

**4.3. Materials and Method**

**4.3.1. Analytical size exclusion chromatography for HMWS determination**

HMWS were quantified using size exclusion chromatography. The method used a Waters Acquity UPLC system (Milford, MA) with a Waters BEH200 1.7 µm (4.6 mm x 150 mm) column (Milford, MA). The column was equilibrated with 1x phosphate buffered saline at 0.3 mL/min. Samples were thawed and centrifuged at 13000 g for 15 minutes then loaded to 20 µg with a 100 µL maximum injection volume. Protein elution was monitored by absorbance at 280 nm. Peak integration was performed to quantify percent HMWS, monomer and LMWS. Based on in-line light scattering detector data, the HMWS peak contained dimeric and multimeric
species, and the LMWS contained monomer fragments with an average molecular weight of 85 kDa. Bovine serum albumin (BSA) and a drug substance reference standard were analyzed as assay controls with the expectation that the relative standard deviation for five injections be less than 5% for the HMWS and main peak areas. Samples were analyzed out of chronological order and assay controls (BSA and reference standard) were analyzed at the beginning and end of the sample set to ensure there was no assay bias over the course of the sample analysis. Error bars were calculated as one standard deviation.

4.3.2. Denaturing size exclusion with mass spectroscopy

Denaturing size exclusion chromatography was used to quantify the levels of covalent HMWS and LCs in samples. An Aglient 1200 system (Santa Clara, CA) with two Phenomenex (Torrance, CA) Biosep 5µm SEC-4000 (300mm x 7.8mm) columns were connected in-series and used to isocratically separate the monoclonal antibody samples. The mobile phase was 25% acetonitrile, 0.1% trifluoroacetic acid, 0.1% formic acid and the column temperature was 40ºC. Under these conditions samples were denatured as they were injected onto the column. Columns were injected with 30-40 µg of protein and absorbance was monitored at 220 nm as well as ESI-MS.

4.3.3. SDS-PAGE gel electrophoresis with coomassie staining

SDS-PAGE gel electrophoresis was performed to determine the relative levels of LC and LC dimer in samples. Invitrogen (Grand Island, NY) Novex 4-20% tris-glycine 1.0 mm precast gels were used for non-reduced and reduced samples conditions. Samples were diluted to 0.05 mg/mL with water and mixed with 1x tris-glycine SDS sample buffer. For reducing conditions, dithiothreitol (DTT) was added to the sample at a final concentration of 50 mM. Gels were
loaded with 1 µg protein and run at 125 V for 90-100 minutes. Following electrophoresis gels were removed from the cassettes and incubated for 5 minutes three times in deionized water. Gels were then stained using Invitrogen (Grand Island, NY) SimplyBlue Safestain with 2% NaCl overnight with shaking.

4.3.4. Protein concentration determination

Protein concentrations were determined using analytical-scale protein-A affinity chromatography. The method used a high performance liquid chromatography (HPLC) Agilent 1100 system (Santa Clara, CA) consisting of a quaternary gradient solvent delivery system, ultraviolet light detector monitoring at 280 nm, autosampler with cooling capability and an Applied Biosystems (Grand Island, NY) POROS A/20 protein-A column (4.6 mm x 50 mm).

Prior to sample analysis the thawed samples were centrifuged at 13000 g for 5 minutes. The supernatant was then transferred to HPLC vials. The column was equilibrated at 3 mL/min using 20 mM tris, 150 mM NaCl (pH 7.0), the sample was loaded to 9.5 – 210 µg protein, washed using 20 mM tris, 150 mM NaCl (pH 7.0) then eluted with 0.22 M acetic acid, 150 mM NaCl (pH 2.6). A purified protein standard was analyzed at various dilutions to generate a standard curve. The standard curve was used for calculating the sample protein concentrations.

4.3.5. Real time quantitative polymer chain reaction

Real time quantitative polymerase chain reaction (RT-qPCR) was used to quantify the heavy chain and light chain mRNA levels on cells from the cell bank vials for the two clonal cell lines. The primers and probes were designed against the mRNA coding sequence for each target. Three independent RNA samples were prepared and analyzed in triplicate for each clone. b-Actin probe
was used to normalize the signal. Reactant concentrations are shown in Table 3. A QuantStudio™ 7 Flex System was used to measure the Ct for the samples.

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>LC</th>
<th>b-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer conc</td>
<td>100 nM</td>
<td>300 nM</td>
<td>300 nM</td>
</tr>
<tr>
<td>Reverse primer conc</td>
<td>900 nM</td>
<td>900 nM</td>
<td>900 nM</td>
</tr>
<tr>
<td>Probe conc</td>
<td>400 nM</td>
<td>400 nM</td>
<td>200 nM</td>
</tr>
<tr>
<td>Mg2+ conc</td>
<td>5 mM</td>
<td>4 mM</td>
<td>3 mM</td>
</tr>
<tr>
<td>RNA input</td>
<td>10 ng</td>
<td>10ng</td>
<td>10 ng</td>
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</tbody>
</table>

Table 3. RT qPCR optimized reactant concentrations

4.3.6. Cell lines, cell culture media and cell culture shake flask experiments

mAbs were produced using two Chinese hamster ovary (CHO) cell lines. The cell lines were clones (clone 40 and clone 59) derived from the same parental cell line expressing the same mAb. The clones were selected to have similar levels of mAb protein concentration at the end of cell culture but with different HMWS concentrations. Clone 40 was associated with a higher aggregation propensity compared to clone 59. Cell culture operations were performed as previously described.

4.3.7. Fractionation of mAb assembly species

Samples containing purified and concentrated mAb or mAb HMWS were prepared from mAb cell culture broth, processed by centrifugation to remove cells, and stored frozen prior to use. After thawing, the cell-free cell culture broth was purified using preparative protein-A chromatography, size-exclusion chromatography (SEC) and centrifugal filter concentrators, as described (Russell 2015, submitted). The protein concentrations in the resulting samples were
0.3-0.9 g/L for HMWS fraction, and 15-16 g/L for monomeric mAb samples. The HMWS fraction contained 10-20% monomeric mAb, with the balance HMWS, whereas the purified monomer samples contained greater than 80% monomeric mAb.

4.3.8. Preparation of mAb-Free spent medium samples

In order to remove background mAb species, spent cell culture medium was incubated with protein-A resin to bind free mAb species which would include monomer, LMWS and HMWS. mAb Select Sure (GE Healthcare, Pittsburgh, PA) was first aseptically prepared by rinsing three times with 1x phosphate buffered saline. Resin rinsing was performed by centrifuging the resin at 5000g for 5 minutes then decanting the supernatant and adding fresh buffer at approximately 50% by volume. After the last buffer rinse, the resin was washed once then resuspended in cell culture medium at 75% v/v resin to slurry volume.

Spent medium from day three cell cultures was treated with protein-A resin by adding 0.1 mL resin slurry per mL spent medium. Resin was incubated in the spent medium for 30 minutes with intermittent mixing. After incubation, the protein-A treated spent medium was centrifuged at 1000g for 5 minutes. The spent medium supernatant was then aseptically transferred into 250 mL shake flasks for use in stability studies. The resin pellet was then resuspended in 100 mM sodium acetate, pH 3.85 buffer (0.65 mL resin/mL total volume) to elute the bound mAb species, after 10 minutes of incubation in the buffer the solution was centrifuged (2000g for 5 minutes) and the supernatant containing the eluted protein-A mAb species was sampled for SDS-PAGE analysis. All test conditions were performed in triplicate.
4.3.9. Monoclonal antibody stability studies

To determine mAb stability in the absence of cells, cell cultures were maintained for three days of protein production after which the broth was centrifuged to remove cells. The supernatant, was used as the solution for stability studies. For some test conditions solutions containing purified and concentrated monomer were “spiked” using a 0.22 µm syringe filter and injected directly into shake flasks in a laminar flow hood. Flasks were placed in an incubator at 36°C with 6% CO2 with rotation at 150 RPM. Samples were taken on days 0, 3, 6, 8 and 10 of the incubation period and stored frozen at -70°C.

4.4. Results

4.4.1. Intracellular LC and HC mRNA levels

Based on the literature suggesting a correlation between intracellular LC:HC ratio and extracellular HMWS formation, we quantified the LC and HC mRNA levels in the two cell lines. Result show that clone 40 has higher mRNA HC levels compared to clone 59. The ratio of LC to HC was calculated to be 1:3 for clone 40 and 1:2 for clone 59.
Figure 13. HC and LC levels by RT-qPCR. Results are normalized to clone 40 HC. Standard deviation is based on three samples run in triplicate.

4.4.2. LC species composition of mAb Aggregates

Our previous work suggested that in our model system, mAb-related components (mAb, mAb LMWS or mAb HMWS) that were present in spent cell culture media contributed to extracellular protein aggregation. To investigate which mAb species might be involved in aggregation, we prepared purified and concentrated pools of HMWS from clone 40 and clone 59 cell cultures and analyzed them by SDS-PAGE gel electrophoresis and denaturing SEC to determine their compositions of covalent HMWS, half molecule, LC and LC dimers. SDS-PAGE gel electropherograms were collected under oxidizing and reducing conditions to determine their LC composition. Because the purified-concentrated HMWS pools contained 10-20% monomer, the gels show a combination of HMWS (> 80%) and free monomeric mAb (< 20%). The results
(Figure 14) show the extracellular HMWS from clones 59 and 40 are similar in LC concentration.

Figure 14: Reduced SDS-PAGE densitometry results for HMWS produced in cultures of clone 40 (stripes) and clone 59 (solid) isolated after three and ten days of cell culture. Densitometry values were normalized to the drug substance light chain band. Drug substance sample represents a high purity mAb monomer (white dots) and is shown for comparison. Error bars represent the standard deviation on replicate gels and experiments with n=4-6 for each sample type. T-test of the cell culture day effect on LC densitometry was statistically significant (p<0.05) for clone 59 and 40 combined data set and clone 59 alone. Culture day was not statistically significant for clone 40 data independently.

Denaturing SEC was also used to assess the composition of the HMWS pools from clone 59 and 40. Under denaturing non-reducing conditions, HMWS in cultures of clone 59 have a slightly lower proportion of covalent HMWS compared to HMWS produced by clone 40 (Table
4). Secondly, the results shown in Figure 15 indicate that the non-covalent portion of the clone 40 HMWS is enriched in both LC and LC dimer.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction (%) of Covalent HMWS</th>
</tr>
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<tbody>
<tr>
<td>Day 3 Clone 59 HMWS</td>
<td>31</td>
</tr>
<tr>
<td>Day 3 Clone 40 HMWS</td>
<td>42</td>
</tr>
<tr>
<td>Day 10 Clone 59 HMWS</td>
<td>52</td>
</tr>
<tr>
<td>Day 10 Clone 40 HMWS</td>
<td>61</td>
</tr>
</tbody>
</table>

Table 4. Denaturing SEC analysis of HMWS isolated from clone 59 and 40 cell culture after ten days of cell growth.
Figure 15: Denaturing SEC results for the non-covalent portion of the HMWS sample. Clone 59 (green) and clone 40 (red) HMWS were isolated after three and ten days of cell culture then purified and concentrated prior to analysis. Results show the percent of non-covalent HMWS composed of LC and LC dimer (diagonal lines) and monomer (solid). No free HC were detected. Error bars represent the standard deviation for multiple sample preparations and injections (n=3). Results were normalized to the molecular weight ratio between the mAb species and monomer.

4.4.3. Stability studies

Stability studies were performed to assess the dependence of extracellular aggregation on extracellular LC and LC dimer concentrations. As was previously shown, protein aggregation can occur in the absence of cells under cell culture conditions when the spent broths contain background mAb and mAb-associated species. HMWS levels measured by SEC increased as a function of incubation time (Figure 16). Under these conditions, mAb produced by clone 40 has a higher propensity to aggregate.
Non-reduced SDS-PAGE gel electrophoresis densitometry was used to quantify the free LC and LC dimer in the supernatant from the stability study cell culture samples. Results show that there is an increase in LC dimer for clone 40 over the ten day stability hold (Figure 17a). Clone 59 showed a decrease in LC dimer during the stability hold. It was expected that with the formation of LC dimers in the clone 40 spent medium that the free LC levels would decrease but analysis by SDS-PAGE densitometry was inconclusive in that only one out of two experimental conditions showed a decrease in free LC during the ten day incubation (Figure 17b). The increase in LC observed for one of the clone 40 conditions could be due to insoluble aggregate disassociation. It is unlikely that the increase in LC would be due to monomer disassociation based on the stability of the monomer under cell culture conditions.
Figure 17. Non-reduced SDS-PAGE densitometry results for clone 40 (dotted line) and clone 59 (solid line) stability study using spent medium isolated after three days of cell culture. Results were normalized to day 0. Error bars represent the standard deviation for two experimental conditions analyzed on separate gels. A) LC dimer B) free LC represented as individual experimental conditions with duplicate conditions for both clones.

Figure 18 confirms the correlation that for clone 40 the extracellular HMWS level increases linearly with extracellular LC dimer levels over the ten day stability hold. Clone 59 mAbs do not show a correlation between LC dimer and HMWS formation in spent medium when monitored for ten days in spent medium.
Figure 18. Relationship between the HMWS measured by SEC and the non-reduced SDS-PAGE LC dimer densitometry for clone 40 (open circle) and clone 59 (solid circle) spent media stability studies. LC dimer band densitometry was normalized to the drug substance LC band. Values represent an average of two experimental conditions. Solid line represents the linear regression for the clone 40 LC dimer results with an r-squared value of 0.868. Linear regression for clone 59 data resulted in an r-squared value of 0.275, line not shown.

4.4.4. Pro-A treated spent medium stability studies

Based on the aggregate characterization and LC kinetics during the spent medium stability hold a study was performed to treat the spent medium to remove the background mAb species. Stability studies were initiated using cell-free cell culture spent medium that had been aseptically treated with protein-A resin.

Prior to performing the stability study the protein-A treatment was tested on spent medium to confirm that mAb was being removed from the spent medium. Samples of spent medium before and after protein-A treatment were analyzed by protein-A HPLC to quantify the
mAb present. Treatment of spent medium reduced the mAb concentration by approximately 75%. Additionally, to confirm that the protein-A treatment removed LC and LC dimer, the resin was recovered from the spent medium by centrifugation. The resin pellet was incubated with a low pH buffer to elute bound mAb species. The protein-A resin elute was analyzed by non-reduced SDS-PAGE gel electrophoresis. Figure 7 shows the SDS-PAGE gel image, lanes 6-8 noted as ‘pro-A elute’ show that the protein-A elute contained both LC and LC dimer. Due to the unknown protein concentration of the post-protein-A supernatant sample, the samples were loaded to a fixed volume not a fixed protein quantity (micrograms), therefore band densitometries cannot be compared between lanes.

Figure 19. Non-reduced SDS-PAGE gel electrophoresis with coomassie blue stain. Clone 59 spent medium after ten days of cell culture was treated with protein-A resin. Lane 2 represents the spent medium prior to protein-A treatment. Lanes 3-5 represent the protein-A treated spent medium, in triplicate. Lanes 6-8 represent the protein-A resin elute, in triplicate. Lane 9 represents drug substance as a control.

Figure 20 shows the SEC results from the stability hold. Protein-A treated spent medium that was spiked with monomer did not show an increase in HMWS for either clone.
Figure 20. HMWS concentration measured by SEC. Clone 40 (open circles) and clone 59 (solid circles) spent medium from a three day cell culture was spiked with clone 40 and 59 monomers, respectively, and monitored for ten days.

4.5. Discussion

In previous studies it was observed that mAb proteins would aggregate in cell culture spent medium in the absence of cells but in the presence of background mAb species. Conversely, it was shown that the mAb monomers were stable in fresh medium and a null cell line spent medium where background species were absent. These results suggested that the background mAb species are factors in causing protein aggregation in cell culture spent medium. Literature has linked intracellular LC and HC proportions to extracellular aggregation propensity and attributed it to an increase in extracellular misfolded mAbs and mAb fragments [32-34, 36-39]. The rationale for this is that when HC is in excess inside of the cell it triggers a stress response pathway that results in inefficient protein folding and secretion of misfolded mAb and mAb fragments.
Real time qPCR results for the mRNA HC and LC levels for the two clonal cell lines used in the present studies show that clone 40 has a lower LC:HC ratio compared to clone 59. This suggests that there might be a higher proportion of intracellular HC peptide accumulated in the clone 40 cells compared to clone 59 cells. Although mRNA levels are not always indicative of the intracellular peptide levels, our results are in agreement with the observed trend between LC:HC ratio and extracellular aggregation propensity. Following the current rationale for the correlation between intracellular HC levels and extracellular HMWS formation, it would be presumed that clone 40 is under a cellular stress response and the folding and secretion processes are overloaded resulting in secretion of aggregation prone mAb fragments.

Based on the existing literature and our previous study results, the HMWS produced during cell culture of two clonal cell lines were analyzed to determine their mAb species content, specifically LC and LC dimer as LC is readily secreted from the cell and prone to aggregation. HMWS were isolated after three and ten days of cell culture production. Reduced SDS-PAGE gel electrophoresis densitometry was used to assess the LC levels for clone 40 and clone 59 HMWS samples. Figure 14 shows that the aggregates from both clones had similar levels of LCs. As the LC levels on day 10 were similar to drug substance (highly purified monomers) it cannot be concluded that the day 10 clone 40 HMWS are necessarily enriched in LC using SDS-PAGE densitometry.

Denaturing SEC was used to assess the LC and LC dimer levels in the non-covalent aggregate population for the clone 40 and clone 59 HMWS samples. The HMWS LC composition of clone 40 aggregates is enriched in LC compared to clone 59 aggregates. The clone 40 HMWS contained approximately 33% LC and LC dimer and 47-56% monomer with
the balance as half molecule. This high proportion of LC species within the aggregates suggests that LC species are participating in aggregate formation for clone 40.

To further investigate a LC mediated aggregation pathway, LC and LC dimer levels were monitored over the course of a ten day stability hold using spent media from both clones 59 and 40 cell cultures isolated after three days of cell culture production. LC and LC dimer levels were determined using densitometry on non-reduced SDS-PAGE gels. Figure 17a shows that clone 40 spent medium supported an increase in LC dimer. The concurrent increase in extracellular LC dimer and increase in HMWS supports a hypothesis that the LC dimerization process is a step in HMWS formation for clone 40. The formation of LC dimer and HMWS containing LC and LC dimer should result in a decrease in free LC during the stability hold. Figure 17b shows that one out of two experimental conditions has an observed decrease in LC in the spent medium during the ten day stability hold for clone 40. The one aberrant condition showing an increase in LC could be due to changes in the insoluble aggregate population that was not analyzed. Clone 59 spent medium results did not show a correlation between increasing LC dimer and HMWS indicating that the aggregation pathway is not predominately using LC and LC dimers for aggregate formation. The observation that two clonal cell lines have different predominate aggregation pathways was previously observed and reported by Ho et al. [39].

The combination of HMWS characterization results showing that HMWS are enriched in LC species and the concurrent increase in LC dimer and increase in HMWS supports an aggregation pathway that uses LC and LC dimers to form HMWS for clone 40. LC species mediated aggregation should then be dependent on extracellular LC species being present such that removal of mAb related species should decrease aggregation. To test this theory spent medium was treated using protein-A resin to remove free mAb species that included monomer,
HMWS, LC and LC dimer. The protein-A ligand does not have a strong binding affinity for free LC so the treated spent medium still contains some level of extracellular free LCs and LC dimer (as confirmed with non-reduced SDS-PAGE gel electrophoresis in Figure 19). A stability study was performed using the treated spent medium whereby previously purified mAb monomers were spiked into the spent medium. Under the conditions tested, the mAb monomer was stable and no increase in HMWS was observed (Figure 20) for either clone. From these results, it appears that protein-A treatment of the spent medium can inhibit aggregation under cell culture conditions. More specifically, the results show that decreasing the level of residual free LCs and LC dimers through protein-A treatment correlated with a reduction in aggregation.

4.6. Conclusions

In these studies, we investigated the role of extracellular LC species in extracellular HMWS formation during cell culture using two clonal cell lines that expressed the same monoclonal antibody but exhibited different aggregation propensities. Several observations were made regarding the aggregation of mAbs from a cell line with higher aggregation propensity (clone 40) under cell culture condition.

First, the clone 40 HMWS were enriched in LC and LC dimer. The composition of the HMWS indicates that free LC and LC dimer are involved in HMWS formation. Second, LC dimer increased concurrently with HMWS formation. These results support a hypothesis that the LC dimerization process is a step in HMWS formation. Third, mAb monomer was stable in the protein-A treated spent medium. The stabilizing effect of the protein-A treatment suggests that the resin removed or decreased the concentration of a mAb species that is necessary to support HMWS formation. These observations support a proposed aggregation pathway that is mediated
by extracellular LC and LC dimers that can be removed by protein-A resin treatment to reduce aggregation. The results presented enhance the understanding of cell culture aggregation and expand on the previous findings showing that cellular stress response causes an increase in aggregation prone misfolded and fragmented mAb species.
Chapter 5: Conclusions

5.1. Analysis of HMWS in cell culture solution by SEC

In order to investigate aggregation in cell culture an analytical method was developed to resolve HMWS from monomer and cell culture medium components in cell culture solutions. The functionality of the method to resolve the HMWS was confirmed using in-line light scattering. The results from the light scattering data showed the molecular weights correctly corresponded to the expected values for HMWS, monomer and LMWS. Additionally, the cleaning method was rigorous enough to handle the complex sample matrix and prevented column fouling, supporting a column lifetime of greater than two years. The method performance was confirmed for each sample set by testing bovine serum albumin in replicate. The acceptance criteria were main peak area ($\leq 10\% \text{RSD}$) and main peak retention time ($\leq 5\% \text{RSD}$). These criteria were consistently met showing a robust method over the lifetime of usage.

5.2. Extracellular mAb aggregation

A small scale cell culture model was developed for investigating mAb aggregation in cell culture. The model was based on a 250 mL shake flask operation using chemically defined medium. Two Chinese hamster ovary cell line clones were selected that both expressed the same monoclonal antibody. Based on results generated during clone screening clone 40 had a higher aggregation propensity compared to clone 59. This observation was confirmed by cultivating the cell lines in the small scale shake flask model and analyzing the cell culture supernatant by SEC.

To assess if the aggregation was occurring intracellularly or extracellularly the cells were removed from the cell culture by centrifugation and the supernatant (spent medium) was incubated under cell culture conditions for ten days. SEC was used to analyze the HMWS
concentration in the incubating spent media. The results showed an increase in HMWS during this incubation period indicating that the aggregation was occurring extracellularly.

5.3. mAb stability under cell culture conditions

To identify factors that correlated to HMWS formation several stability studies were performed to investigate the mAb stability under cell culture conditions. Using a step-wise approach factors in HMWS formation were eliminated.

Initial stability of the monomer mAb was assessed in 1X PBS buffer under cell culture conditions (i.e. shaking at 36°C). Incubation of the mAb monomer for ten days did not generate HMWS. This result indicates that the temperature and agitation alone do not contribute to the aggregation observed in the cell culture.

Monomer mAb stability was next assessed in fresh medium under cell culture conditions. Again, incubation of the mAb monomer for ten days did not generate HMWS. This result eliminates the media components as contributing factors in the HMWS formation.

Lastly, monomer mAb stability was assessed in a null cell line spent medium. The null cell line does not express the mAb but does produce typical cell by-products and metabolites. After ten days of incubating the mAb monomer in the null cell line spent media there was no increase in HMWS for clone 40 monomer and only a minor increase was observed for clone 59. The increase observed for clone 59 was not significant enough in magnitude to explain the aggregation seen in cell culture. These results eliminate cell by-products and metabolites as factors in HMWS formation during cell culture.

The combination of conclusions suggests that the HMWS formation is caused by a factor that is specific to the clonal spent media such as background mAb species.
5.4. Modulation of HMWS levels in spent medium

A cross-spike experiment was performed that incubated clone 40 aggregates in clone 59 spent medium. Conversely, clone 59 aggregates were incubated in clone 40 spent medium. Incubation of clone 40 aggregates in clone 59 spent medium resulted in lower HMWS formation than when the aggregates were incubated in clone 40 spent medium. This indicates that the clone 40 spent medium promotes the aggregation propensity. This was confirmed by the increased HMWS formation of clone 59 aggregates in clone 40 spent medium.

Differences in the two clonal spent media that could be factors in the HMWS formation would include the background mAb species both the types (i.e mAb fragments, misfolded monomers or HMWS) and concentrations.

5.5. Light chain content of HMWS

In an effort to understand which spent medium background mAb species might be contributing to the HMWS formation the composition (light chain, light chain dimer and half molecule) of the aggregates was determined by denatured SEC and SDS-PAGE gel electrophoresis. HMWS isolated after ten days of cell culture from clone 40 were enriched in light chain and light chain dimer compared to clone 59 HMWS. This composition difference between the two clone HMWS suggests that light chains are involved in the HMWS formation as it correlates with the aggregation propensity.

5.6. Light chain kinetics during HMWS formation

The HMWS characterization results suggested that light chains might be involved in the HWMS formation. To assess this hypothesis the light chain and light chain dimer concentrations
were measured on samples from a previous spent media stability study. Spent media was generated from clone 40 and 59 cell cultures after three days of cell growth by centrifugation to remove the cells. The spent media were then incubated under cell culture conditions for ten days. During this incubation, clone 40 showed an increase in HWMS with a decrease in light chain and a correlating increase in light chain dimer. These results suggest that the clone 40 HMWS formation is via a light chain dimerization pathway. Clone 59 did show a decrease in light chain but no increase in light chain dimer suggesting that the clone 59 monomers are aggregating under a different pathway compared to clone 40.

5.7. Impact of removing background mAb species on aggregation propensity

Protein-A resin selectively binds through the Fab region on the mAb molecule and therefore has specificity for binding mAb monomer and populations of mAb LMWS and HMWS but has weak binding affinity for light chains. To further investigate which mAb species might be involved in the HMWS formation the spent media from clone 40 and clone 59 were incubated with protein-A resin. The resin was removed by centrifugation and the treated spent media were incubated with mAb monomers. Neither clone 40 or 59 monomers aggregated in the protein-A treated spent medium. This indicates that the protein-A resin removed a reactant needed for HMWS formation. As the light chains would not have been exclusively removed by the resin treatment the results suggest that there is a mAb species, most likely containing a Fab domain, that is also involved in the HMWS formation. The results show that through treatment of the spent medium with protein-A resin the aggregation propensity can be reduced for both clones.
Chapter 6: Bibliography

1. Reichart, J., Antibodies to watch in 2014, mAbs, 6, 2014.


70. Gabrielson, J., Arthur, K. , Measuring low levels of protein aggregation by sedimentation velocity, Methods 54, 2011.
Appendix A: Aggregate Characterization

Elucidation of protein characteristics associated with the cell culture aggregates provides information on the potential aggregation mechanism. Several analytical methods were used to explore the aggregate characteristics including DTNB for assessment of free-thiol content, reduced/non-reduced SDS-PAGE gels to quantify the covalent, heavy chain and light chain portions of the aggregates, peptide map to determine the protein heterogeneity, FTIR to determine secondary structural differences, AUC for aggregate sizing and microflow imaging for particle size distribution. Methods for SDS-PAGE gel electrophoresis and aggregate and monomer sample generation were previously described in chapters 2-4.

A.1 Methods

A.1.1 DTNB Assay

Free thiol was quantified using a 5,5’-dithio-bis-(2-nitrobenzoic acid) (Ellman’s) reagent in solution with ultraviolet light absorbance detection. A 0.1M Tris-HCl, pH 8.0 reaction buffer was used for sample dilutions. A stock solution of 10 mM Ellman’s reagent was prepared using 0.1M Tris-HCl buffer. Reacted samples were prepared by combining 50 µL Ellman’s reagent solution, 2.5 mL reaction buffer with 250 µL sample. Samples were incubated for 15 minutes at room temperature. Absorbance was measured using a Nanodrop spectrophotometer at 412 nm. The concentration of free sulfahydrl was calculated using a molar extinction coefficient for TNB of 14, 150 M\(^{-1}\) cm\(^{-1}\).

A.1.2 Peptide map

Purified IgGs were digested by endoproteinase Lys-C (Liu et al., 2009). Briefly, a small IgG sample volume containing 100 µg of protein was denatured in guanidine (6.5 M final
concentration) for 2 h at 37 °C. The sample was then diluted with 8 M urea, 0.2 M phosphate, 40 mM NH2OH, pH 7.0 and water to achieve a final concentration of 4 M urea. This dilution step maintains the IgG in the denatured state while lowering the GuHCl concentration (<0.5 M final concentration) to prevent Lys-C inhibition. Lys-C was added to achieve a substrate: Lys-C ratio of 20:1 (w/w). The protease digest proceeded at 37 °C for 20 h. Sample freezing was used to quench the reactions.

The Lys-C digested IgG samples were analyzed by LC/MS/MS on an Agilent 1200 SL HPLC system directly connected to a Thermo Scientific LTQ-Orbitrap high-resolution mass spectrometer. Peptides were resolved on a Waters 1.7 μm particle column (BEH300 C18, 2.1 mm × 100 mm) at 50 °C, with a gradient of 0.5–20% acetonitrile in 40 min, followed by 20–40% acetonitrile in 80 min, with 0.04% trifluoroacetic acid (TFA) in each mobile phase, at a flow rate of 0.3 mL/min. The mass spectrometer was set up to acquire one high-resolution full scan at 60,000 resolution (at m/z 400), followed by three concurrent data-dependent MS/MS scans of the top three most abundant ions, with dynamic exclusion, using collision-induced dissociation (normalized collision energy 35%). The dynamic exclusion duration was set at 12 s, which is slightly shorter than the width of a typical chromatographic peak to ensure at least one high-quality MS/MS scan for each major ion. Singly charged ions were excluded from MS/MS. About 100 μL (10 μg protein) of each digest was injected into the LC/MS system for analysis. The LC/MS/MS data were processed by MassAnalyzer for automated identification and quantification of different glycopeptides (Zhang, 2009). Structures were assigned based on a combination of accurate mass, MS/MS fragmentation patterns, and biosynthetic restrictions.
A.1.3 FTIR

FTIR of the HMWS was performed using a Bruker Vertex 70 FTIR spectrometer equipped with an AquaSpec transmission cell and a LN-MCT photovoltaic detector. Data was acquired and analyzed with OPUS v. 6.5 software. Spectra were recorded at room temperature with 128 scans interferogram with a 4cm⁻¹ resolution. Reference spectra were captured under the same conditions and subtracted from the sample spectrum.

A.1.4 Archimedes particle sizing

Particle sizing was used to determine the particle size distribution of the HMWS. An Affinity Biosensors Archimedes A fluid density of 1.006 g/cc was assumed. The sensor ID was Micro-A889 which has a range of 200 nm to 6 µm. Data was captured and analyzed with Particle Lab software version 1.8.1085 following the method described in the vendor user manual.

A.1.5 Analytical ultracentrifugation

Analytical ultracentrifugation method was previously described in Gabrielson 2011 [70].

A.2 Results

A.2.1 Free thiol

Results presented below show that the monomer free-thiol levels are constant from day 3 to day 10 cell culture. The clone 59 HMWS sample shows a lower free-thiol content on day 3 compared to the day 10 HMWS and monomer samples. Whereas the clone 40 HMWS shows an increase in free-thiol on day 3 HWMS sample suggesting that HMWS from day 3 cell culture may be less stable due to exposed thiol groups.
Figure 21. Free-thiol levels as measured by DTNB assay for concentrated-purified monomer and HWMS samples that were isolated from cell cultures after three or ten days.

A.2.2 Peptide Map

Analysis of the peptide maps for the monomer and aggregate suggests variations in the glycan structure of the monomers that are involved in the HMWS. The most significant difference was in the high mannose content (Table 4) of the clone 40 HMWS compared to its monomer showing enrichment in the HMWS.
<table>
<thead>
<tr>
<th></th>
<th>Clone 40</th>
<th>Clone 59</th>
<th>Drug Substance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HMWS</td>
<td>Monomer</td>
<td>HMWS</td>
</tr>
<tr>
<td></td>
<td>Percent Glycan Species (%)</td>
<td>Percent Glycan Species (%)</td>
<td></td>
</tr>
<tr>
<td>N300+A1G0F</td>
<td>8.64</td>
<td>6.31</td>
<td>7.92</td>
</tr>
<tr>
<td>N300+A1G1F</td>
<td>2.28</td>
<td>1.81</td>
<td>4.53</td>
</tr>
<tr>
<td>N300+A2G0</td>
<td>1.2</td>
<td>1.3</td>
<td>3.07</td>
</tr>
<tr>
<td>N300+A2G0F</td>
<td>36.49</td>
<td>52.2</td>
<td>30.83</td>
</tr>
<tr>
<td>N300+A2G1</td>
<td>2.36</td>
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<td>5.79</td>
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<tr>
<td>N300+A2G1F</td>
<td>7.17</td>
<td>13.47</td>
<td>6.75</td>
</tr>
<tr>
<td>N300+A2G2F</td>
<td>1.23</td>
<td>2.06</td>
<td>1.83</td>
</tr>
<tr>
<td>N300+M5</td>
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<tr>
<td>N300+M6</td>
<td>10.48</td>
<td>4.71</td>
<td>7.45</td>
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<tr>
<td>N300+M7</td>
<td>7.83</td>
<td>2.77</td>
<td>6.96</td>
</tr>
<tr>
<td>N300+M8</td>
<td>4.19</td>
<td>1.6</td>
<td>5.39</td>
</tr>
<tr>
<td>N300+M9</td>
<td>1.49</td>
<td>0.5</td>
<td>2.89</td>
</tr>
<tr>
<td>N300+Unglycosylated</td>
<td>1.19</td>
<td>1.1</td>
<td>2.71</td>
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<tr>
<td><strong>Total High mannose</strong></td>
<td><strong>39.57</strong></td>
<td><strong>18.54</strong></td>
<td><strong>36.94</strong></td>
</tr>
</tbody>
</table>

Table 5: Peptide results by non-reduced Lys-c for concentrated and purified monomers and HMWS from clone 40 and 59 cell cultures. Cell cultures were clarified and purified after ten days of cell growth. Drug substance is shown as a control representing a highly purified monomer.
A.2.3 SDS-PAGE Gel Electrophoresis with Coomassie Stain

Figure 22. SDS-PAGE gel showing HMWS and monomer samples isolated and purified from clone 59 and clone 40 cell cultures after three or ten days of cell culture production. Lane designation is the same on the non-reduced and reduced gels: 1) drugs substance 2) Day 10 clone 40 monomer 3) Day 10 clone 40 HMWS 4) Day 10 Clone 59 monomer 5) Day 10 clone 59 HMWS 6) Day 3 clone 40 monomer 7) Day 3 clone 40 HMWS 8) Day 3 clone 59 monomer 9) Day 3 clone 59 HMWS 10) SeeBlue pre-stained standard molecular weight marker

Figure 23. SDS-PAGE gel (same as shown above) with spyro orange image filter to improve visualization of LMWS.
Figure 24. AUC overlay for concentrated-purified monomer and HWMS samples that were isolated from cell cultures after ten days. All samples were normalized to monomer peak max.
A.2.5 FTIR

Figure 25. FTIR for concentrated-purified HWMS samples that were isolated from cell cultures after ten days from clone 40 (pink) and clone 59 (green)
A.2.6 Microflow imaging

Figure 26. Archimedes spectrum for concentrated-purified clone 40 HWMS sample that was isolated from cell culture after ten days

Figure 27. Archimedes spectrum for concentrated-purified clone 59 HWMS sample that was isolated from cell culture after ten days
Appendix B: Size exclusion chromatography assay development

Initial work was performed to develop a size exclusion chromatography assay that could resolve HMWS directly in cell culture spent media samples. Previous to this work, aggregation in cell culture was assessed on protein-A chromatography purified cell culture samples. This additional purification step can impact the HMWS profile of the cell culture sample and render it not representative (Chapter 3). These results confirmed the need to develop an SEC method that could be used to directly analyze cell culture samples without purification.

The SEC method was developed on a Waters Acquity UPLC chromatography system for high speed analysis utilizing a 1X phosphate buffered saline (PBS) running buffer for efficiency and cost savings. After the method was optimized for HMWS resolution, the peak was fractionated to confirm that the HMWS peak was indeed the antibody. The HMWS peak and monomer peak were run on a protein-A chromatography column to confirm that they bound to the resin and were indeed the antibody of interest. 26 shows the chromatograms for SEC and protein-A analyses. Additionally, light scattering was used to confirm the molecular weight of the HMWS, monomer and LMWS peaks (see Chapter 2). The HMWS peak had a molecular weight of ~364,000 Da, the monomer peak had a molecular weight of ~155,000 Da and the LMWS peak was ~85,000 Da. The monomer theoretical molecular weight is 147,000 Da which matches closely with the light scattering value. The HMWS peak is approximately twice the monomer but likely contains higher order aggregates or a heterogeneous isoforms that shift the average molecular weight higher. The LMWS peak represents a half molecule comprised of a light chain and heavy chain. Two assay controls were used to confirm assay performance. Bovine serum albumin (BSA) main peak results had a percent of main peak and main peak retention time of less than 2% relative standard deviation and main peak area with a relative
standard deviation of less than 10%. Drug substance (highly purified monomer) was also used as a control for the monomer. The monomer peak retention time of the samples was within 3% of the drug substance average retention time. These criteria were used as assay acceptance criteria for all sample sets.

Figure 28. Panel A shows the SEC chromatogram where F1 represents the HMWS and F2 represents the monomer. Panels B and C show the protein-A chromatograms for the respective fractions. In both chromatograms the protein bound to the resin and was eluted confirming that it is primarily antibody in composition and based on the high levels is specifically the antibody of interest. Samples analyzed and presented in panel A were conditioned media from day 3 production flasks from the Clone 40 cell line (n=4).
Appendix C: Effect of cystine/cysteine addition to spent media

To investigate the impact of free-thiol on cell culture aggregation cysteine and cystine were used to adjust the redox potential in the cell culture spent medium. Stability studies were performed using spent media isolated after three days of cell culture. The test conditions for the stability study are shown in the table below. Stocks solution of 399 mM L-cystine-2HCl and 700 mM L-cysteine were prepared and used to spike the spent media.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cystine (mM)</th>
<th>Cysteine (mM)</th>
<th>Spent Media Clone Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1.65</td>
<td>59</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>4.95</td>
<td>59</td>
</tr>
<tr>
<td>4</td>
<td>0.64</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>5</td>
<td>1.92</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>1.65</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>4.95</td>
<td>40</td>
</tr>
<tr>
<td>10</td>
<td>0.64</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>11</td>
<td>1.92</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 6. Experimental conditions for cysteine/cystine stability study using clonal spent medium
Stability study SEC results for clone 40 conditions are shown below. The no-spike condition represents the control condition with no adjustment to the cystine/cysteine concentration. The addition of cystine and high levels of cysteine appeared to lower the HMWS compared to the control condition. Clone 59 conditions exhibited no HMWS as measured by SEC.

Figure 29. SEC results from cystine and cysteine conditions using clone 40 day three spent medium
Appendix D: Effect of galactose addition to cell culture media

Previous work by Ho et al has shown a correlation between enriched high mannose proteins and increased aggregation [39]. Based on this observation a stability study was performed under cell culture conditions that modify the high mannose content of the monomer population. To modify the high mannose content of the monomer galactose was added to the cell culture media during production. Spent media from the galactose cell culture production was then used as a stability solution. The spent media flasks were incubated for 10 days and monitored by SEC. No significant change in aggregation propensity was observed with the addition of galactose to the cell culture. These results indicate that changes in the carbohydrate source for glycosylation do not impact the aggregation propensity.

![Graph showing HMWS concentration over stability test days for galactose and control media]

Figure 30. HMWS concentration measured by SEC for spent media stability study using galactose supplemented cell culture media. Study used clone 40 spent media from a three day cell culture.