Aggregation Propensity: Characterization of Monoclonal Antibody Stability

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Aggregation Propensity:

Characterization of Monoclonal Antibody Stability

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Abstract

      The study of aggregation propensity of a monoclonal antibody (mAb) and its sensitivity to applied stresses is believed to correlate with the overall stability of the mAb. As such, the aggregation propensity under various stresses can be used to develop a unique aggregation metric to rank order a panel of mAbs based on their stability. Often in a drug discovery campaign, multiple mAbs may imbue the desired in vivo efficacy, at which point identification of the most developable mAb becomes an important factor to decide on a single candidate for further development. This study focuses on the assessment of the stability of a panel of mAbs, by defining their propensity for aggregation along the native and non-native aggregation pathways. Kosmotrope based solubility evaluates a mAb's colloidal stability, or propensity for native aggregation, while differential scanning fluorescence reports conformational stability, or propensity for non-native aggregation. By combining the conformational and colloidal stability metrics, an overall aggregation propensity profile can be generated for a mAb. To parse out further information on stability, the mAb panel was exposed to a series of stresses, which mimic stresses a mAb based drug would be exposed to during manufacturing and storage. After exposure to stress, the mAb panel was then monitored for change in apparent colloidal and conformational stability. There was no variation in the stability metrics measured, as a function of stress. However, observed precipitation denoted differential sensitivity to the stresses. Combining observational data with the stability

metrics measured, allowed for rank ordering of aggregation propensity, and overall

stability.

Dedication

This effort is dedicated to my family, Melissa and Emmett. You are my motivation.

Without you, this would have been an utter failure.

Acknowledgements

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Chapter I

Introduction

Monoclonal antibodies are becoming a major component of the modern drug landscape. However, mAbs are highly complex biomolecules, which need to maintain their native monomeric conformation to retain efficacy, avoid potential patient immunogenic responses, and comply with regulatory requirements. Thus, methods to monitor mAb stability and predict degradation propensity, such as aggregation, allow for the identification of the most stable drug candidate, and are vital to the success of a drug discovery campaign.

Monoclonal Antibodies and Their Importance in Modern Medicine

Monoclonal IgG antibodies represent one of the fastest growing sections of the pharmaceutical marketplace, with most major pharmaceutical and biotechnology companies developing antibody based therapeutics. One of the many reasons behind mAb popularity is their great versatility, with its relatively stable nature, ability to bind to a variety of targets, and multiple modes of action including incapacitation of the target, host complimented cytotoxicity, and directed drug delivery, in the case of antibody-drug conjugates (Leader, Baca, & Golan, 2008). As such, mAbs have been approved or have approval pending by FDA for treatment of a variety of diseases including multiple types

of cancer, infectious diseases, rheumatoid arthritis, multiple sclerosis, Alzheimer's disease, and other orphan diseases with no current mode of treatment (Reichert, 2013). The discovery of mAbs is supported by a broad range of techniques, including library screening of phage and yeast surface display, panning of B-cells, and hybridoma development (Lu et al., 2012). Significant advances in these techniques have resulted in a robust pipeline of antibodies in development.

The first monoclonal antibody drug was approved by the FDA nearly 30 years ago for immune modulation following transplants. The number of areas in which mAbs are used has increased dramatically over the years. To date, a total of 30 mAbs have been approved for therapeutic use. Of those 30 mAbs, 13 are used in cancer treatment. However, as of early 2013, 29 mAbs are in Phase 2 or 3 clinical trial: 19 of these mAbs are not oncology directed drugs (Reichert, 2013). This showcases the flexible use of mAbs from a therapeutic standpoint. This is due to the ability of a mAb to bind highly specific, targeted epitopes, thus allowing for high efficacy.

A therapeutic mAb engage a specific target through its variable region (Fv). The site on the antibody that makes contact with the target antigen is known as the paratope and site of engagement on the target is known as the epitope. For mAbs whose mechanism of action is mediated by the action of the effector function, interaction with immune cells through the constant region (Fc) is critical. Therapeutic mAbs act through several methods; the mAb can bind to its epitope, thus preventing an activity or function, or through an immune response in which the mAb signals its target for destruction using host complimented cytotoxicity (Lazar et al., 2006). Additionally, mAbs can be used as antibody-drug conjugates, in which the mAb acts as a targeting system, bringing linked

small molecule drugs to the point of need, thus reducing toxicity issues associated with some of the more potent drugs (Corrigan, Cicci, Auten, & Lowe, 2014). As the diversity of applications for mAbs increases, so does the methods used to find and improve these potential drug candidates.

Monoclonal Antibody Discovery

The immune system is responsible for the production of antibodies. In a normal human, there is an estimated 10^{10} differently targeted mAbs (Fanning, Connor, & Wu, 1996). Thus, generation and identification of mAbs with a desired in vivo activity is vital for the development of mAb based drugs. Several different methodologies are currently used for mAb identification, including library display, B-cell panning, and hybridoma development (Chan, Lim, MacAry, & Hanson, 2014; Ribatti, 2014). Library display is the screening of a naïve, immune or synthetic library displayed on phage or yeast surface against the desired antigen. B-cell panning involves the assessment of individual cell production of mAbs against the desired antigen. Depending on the source of library, these methods can have the benefit of generating fully human antibody sequences, which is desirable, as the more human or human-like the primary sequence, the lower the potential for immunogenicity (Ponsel, Neugebauer, Ladetzki-Baehs, & Tissot, 2011). Similar to B-cell screening, the hybridoma development method uses a model organism, generally a rat or mouse, which is injected with the target protein of interest. Antibodies against that target are created and then evaluated ex vivo for the desired characteristics (Ribatti, 2014). These methods of antibody discovery often result in multiple unique

drug candidates that meet the desired efficacy criteria. To facilitate the selection of the best possible candidate for human clinical trials, it is important to develop a good understanding of key development criteria and use that in conjunction with efficacy data.

Characteristics of a Monoclonal Antibody for Development

The goal of a mAb drug discovery program is to identify a candidate with the desired activity profile and an acceptable safety profile that can be reliably produced in large quantities and can be stored over extended period of time. Development of a therapeutic mAb assesses a variety of topics, including production, purification, stability and homogeneity of the product. For a mAb to be produced into a drug, it must be developable, which can be determined by focusing on two categories: manufacturability and stability. Manufacturability encompasses protein expression rates, production purity, resilience to stringent production processes, and compatibility with formulation (Ponsel et al., 2011). Expression rates are a key limit in drug production, as the cost of goods is directly linked to the amount of protein produced by a stable cell line, and as such a low expressing development candidate is likely not a viable drug candidate. Stability can be further broken down into three categories: conformational, colloidal, and chemical, and is the primary focus of this study.

Stability of Monoclonal Antibodies

As with any biologic based drug, purity and stability is vitally important. While mAbs are inherently a stable class of protein, the stability of each mAb varies. A typical dose requires large amounts of mAb, and with the generally desired subcutaneous delivery, the maximum volume is less than 2 mL, resulting in necessary drug formulation concentrations of greater than 50 mg/mL (Tessier, Wu, & Dickinson, 2014). Thus, the stability of a protein based drug in this context is defined as the protein's ability to maintain the desired conformation and activity in solution, at high concentrations, for the shelf life of the protein which is generally one to two years at 2 °C – 8 °C storage (Banks et al., 2012). While mAbs are known for their relatively good stability, these stringent requirements demand that, in addition to extensive efforts to identify the most stable formulation, a construct with a good stability profile is selected early in the drug discovery process.

That mAbs are highly complex molecules contributes to the difficulty of maintaining stability throughout the extensive process of manufacturing, container filling, storage, and administration (Alsenaidy, Jain, Kim, Middaugh, & Volkin, 2014). That same complexity also adds to the difficulty in characterizing mAbs, and proteins in general, and has led to extensive regulations implemented by the Food and Drug Administration, and other regulatory agencies worldwide, including the established guidelines: "Stability testing of Biotechnological/Biological products" (International Conference on Harmonisation, 1996). That mAbs have a generally high degree of sequence similarity does not translate to uniform physicochemical profiles, and as such, stability profiles can vary greatly (Goldberg et al., 2011).

All proteins have a propensity to degrade over time, and it has been proposed that virtually any protein will aggregate in solution if given enough time, unless proteolytic cleavage breaks down the protein, or formulation can slow or stop the process (Roberts, 2007). Aggregation is considered the most common challenge faced when dealing with high mAb concentration formulas, as it can lead to high viscosity solutions, reduce the activity of the drug, and potentially lead to an immunogenic response in the patient (Roberts, 2014; Tessier et al., 2014). Aggregation of a protein drug can also lead to unintended side-effects, such as unintended receptor interaction (Yamniuk et al., 2013).

The presence of aggregated proteins has been known to increase the potential for a patient to develop an unintended immune response, which could target the active monomer. Of approved antibodies on the market, 40% of chimeric, and 9% of humanized antibodies triggered an anti-drug response (Hwang & Foote, 2005). An unintended immune response can result in the drug losing efficacy, especially in the setting of a chronic disease where repeat administration of the mAb may be required. In addition, there is the possibility, albeit very rare, of the aggregated material triggering an autoimmune event, such as red cell aplasia, in which a patient's system begins targeting endogenous proteins (Roberts, 2014). Because of the critical need for stability and the challenges associated with aggregation, it is vital to identify drug candidates early in the discovery process that have an acceptably low propensity for aggregation, thus reducing the potential for failure during clinical development (Yamniuk et al., 2013).

Formulation and Mutagenesis

Inherent stability is highly desirable for any drug candidate. However, it is widely accepted that proteins are not stable in their native folded conformation (Roberts, 2014). Thus, to improve protein stability, extensive efforts are made to stabilize the protein through formulation and/or mutagenesis. Formulation focuses on identifying key characteristics of a solution that will best stabilize the protein. Mutagenesis attempts to identify areas on the protein that may cause stability issues, and address the vulnerability through introduction of mutations in the primary sequence. Both methods have been used and can be used to improve protein stability. However, neither can completely prevent protein degradation, and both are often challenging.

Formulation is a key element of a successful, stable protein drug. By identifying optimal solution conditions such as pH, ionic strength, and excipients, the propensity for degradation can be reduced (Goldberg et al., 2011). Significant time and energy can be invested in evaluating the myriad number of formulation combinations, each potential combination affecting protein stability either subtly or significantly. Formulation is used to improve both the conformational and colloidal stability of a protein through stabilizing not just the native conformation, but potentially stabilizing partially unfolded intermediates, thus preventing aggregation (He et al., 2010). Unfortunately, formulation cannot always improve a protein's stability to the necessary level of a drug product, in which case mutagenesis may help stabilize the protein.

Stabilization through mutagenesis is the process of identifying regions of a protein that may cause stability issues, and then altering key amino acids in those regions

in an attempt to negate the stability issue. A structure based in silico assessment called spatial aggregation propensity (SAP) identifies "hot-spots" on the surface of proteins that have the potential to cause native aggregation, and then models mutations which may neutralize those regions (Voynov, Chennamsetty, Kayser, Helk, & Trout, 2009). Additionally, efforts to identify conformationally unstable regions in the Fab, through in silico modeling, have had mixed success, but ultimately a difficult time identifying stabilizing mutations that do not affect the mAb's binding profile (Lee et al., 2013). Most mAbs have reasonably high sequence similarity, with the exception of the variable region, which is unique to each mAb and is responsible for its binding profile. The variable region of a mAb tends to have the highest risk of inducing aggregation. While mutagenesis studies have been conducted and have shown some success at reducing aggregation without affecting efficacy, a limitation of this approach (besides not always being successful) is that it changes the primary sequence, which necessitates retesting of the mAb for efficacy (Lee et al., 2013; Lu et al., 2012). In silico modeling may be able to identify regions that can cause aggregation, but those regions cannot always be mutated without negatively affecting the mAb, as some mutations may interfere with proper folding and others may affect pertinent residues directly involved with epitope binding (Goldberg et al., 2011; Roberts, 2014). And while the technology behind the SAP analysis will continue to improve, in its current state many believe that due to the diverse number of mechanisms of aggregation, in silico aggregation prediction is limited (Yamniuk et al., 2013). Thus, there are limits to both formulation and mutagenesis as means to generate a highly stable protein based drug.

While both formulation and mutagenesis can help improve the stability profile of a protein, neither can completely quench inherent stability issues in a protein. Thus, it is vital to identify drug candidates with the best stability profile early in the development process, so formulation and mutagenesis, if necessary, can provide the final stabilizing effect for a protein and generate a stable drug.

Protein Degradation

Stability is vital to the successful production of a protein-based drug. However, a protein's stability is affected by multiple pathways as it progresses through production, filling, and storage, prior to delivery to a patient. The mechanisms of degradation can be categorized as either chemical or physical. Chemical degradation in itself can take multiple different pathways, including deamidation, oxidation, proteolysis, and disulfide bond shuffling. Physical degradation has multiple pathways as well, which include structural alterations, aggregation, and precipitation (Alsenaidy, 2014). The degradation pathways are diverse and can have extremely negative effects on proteins. Thus it is imperative to understand an individual protein's propensity to one or more of those pathways, which will better aid in understanding potential pitfalls associated with a drug candidate.

Degradation Pathways

Chemical degradation can be defined as any change in the covalent structure of a molecule. In the case of an extremely complex molecule such as a protein, there are multiple potential points for degradation. As previously mentioned, there are several common routes of chemical degradation, such as deamidation, oxidation, proteolysis, and disulfide bond shuffling. Deamidation is the process of hydrolyzing asparagine and glutamine side chains, which results in degradation products, and potentially enhanced in vivo immunogenicity (Manning, Chou, Murphy, Payne, & Katayama, 2010). Oxidation can be caused by multiple factors, but ultimately involves a reaction with a reactive oxygen species, which can damage the side chains of multiple amino acids (his, met, cys, tyr, and trp). Disulfide bond shuffling involves the reduction of disulfide bonds between cysteine in the native conformation, and then reformation in an incorrectly folded conformation due to incorrect cysteine pairing formation of cystine. Finally, proteolysis can be caused by multiple methods, including oxidation, hydrolysis, and N- and C-terminal residue clipping. There are many variations of these basic chemical degradation pathways. Fortunately, some pathways tend to occur very slowly over a product's lifetime, or can be minimized through protein mutagenesis, formulation, packaging, and/or storage (Manning et al., 2010).

Physical degradation is a key concern in the development of therapeutic proteins, as it is widely accepted that proteins have only a marginal degree of native fold conformational stability (Roberts, 2014). Physical degradation of proteins takes place in several different forms: denaturation, aggregation, and precipitation. Complete denaturation of a protein can be caused by multiple chemical or physical forces, and

results in low solubility aggregates (Kramer, Shende, Motl, Pace, & Scholtz, 2012).

Precipitation is caused either by a protein exceeding its limit of solubility, or by soluble

aggregate-aggregate association, clumping, or the monomeric addition to an aggregate

cluster, otherwise known as a nucleation site, until the aggregate reaches a size at which

it becomes insoluble and forms visible particulate or precipitation (Manning et al., 2010;

Roberts, 2007). Significant efforts are made to minimize a protein's propensity to enter

into the aggregation pathway (Roberts, 2014). Thus, by appropriately screening mAbs

early in the development process, and focusing on aggregation propensity, the efforts

necessary to minimize aggregation through alternate means, such as formulation and

mutagenesis, can be reduced dramatically.

Aggregation

Aggregation itself follows two pathways: non-native state and native state

formation. Native and non-native aggregation pathways represent the primary focus with

respect to physical degradation and the development of protein based drugs.

Native aggregation. Native aggregation is the pathway in which natively folded protein

begins to self-associate. The cause of self-association is a combination of environment

and physical characteristics of the protein itself (Figure 1, Folded 'clusters'). The

environmental factors that affect native state association include pH, ionic strength,

temperature, and presence of other excipients (Kramer et al., 2012). The physical

features of a protein that increase or decrease the likelihood of native state association

include surface hydrophobicity, charge, and the propensity to form β-sheets and α-helixes (Lauer et al., 2012).

The physical characteristics that tend to encourage native aggregation involve uncharged hydrophobic patches along the protein's surface (Roberts, 2014; Yamniuk et al., 2013). The environmental factors involved can encourage or reduce native aggregation propensity. Solutions buffered at or near the isoelectric point (pI) of the protein reduce net charge to near zero, thus reducing repulsive forces, and allowing monomers to attain close proximity. The ionic strength of the solution can also negate surface charges in a manner similar to a protein at its pI (Yamniuk et al., 2013).

Initially, the association of natively folded proteins is reversible, but over time and with the close proximity of other proteins, the entropic penalty for formation of β-sheets with nearby protein strands lessens because the forces that drive proper folding also drive aggregation (Roberts, 2014). Thus, by allowing these proximal associations to sample multiple conformations, a lower energy state may be found. Most often in these situations the conformation formed is a β–sheet, creating a strong, irreversible, non-covalently bound aggregate with other nearby monomers (Caflisch, 2006; Roberts, 2007). This aggregate is generally called a nuclei (Figure 1), and is where the differentiation between native state and non-native aggregation ends, as experimental methods generally cannot distinguish the origin of a nuclei between association before unfolding, or unfolding before association (Roberts, 2007).

Non-native aggregation. Non-native aggregation is the pathway in which the active monomer loses proper conformation and becomes partly unfolded. This partly unfolded

intermediate has core hydrophobic residues exposed, and begins to associate with other partly unfolded monomers through hydrophobic interaction (Figure 1, Partly unfolded monomers). This process is widely believed to be irreversible (Andrews & Roberts, 2007; Banks et al., 2012; Costanzo et al., 2014). Efforts to minimize non-native aggregation include mutagenesis and formulation.

Because mAbs are a large, complex, multi-domain structure, the conformational stability is regarded to be relatively low (Roberts, 2014). There are three predominant regions in which variance in conformational stability can be observed: the constant heavy chain regions 2 (C_H2) and 3 (C_H3), as well as the antigen binding domain (Fab). In general it has been reported that the C_H2 domain tends to show lowest conformational stability, although the Fab can also account for significant conformational instability (Lee, Perchiacca, & Tessier, 2013; Shi et al., 2013). Efforts have been made to stabilize native conformational stability through rational protein design and mutagenesis (Lee et al., 2013; Roberts, 2014). Others have made attempts to stabilize the partly unfolded intermediate prior to irreversible pairing and aggregate formation, through formulation (Costanzo et al., 2014, Goldberg et al., 2011). Ultimately, upon irreversible aggregation and nuclei formation, the distinction between the native and non-native aggregation pathway diminishes.
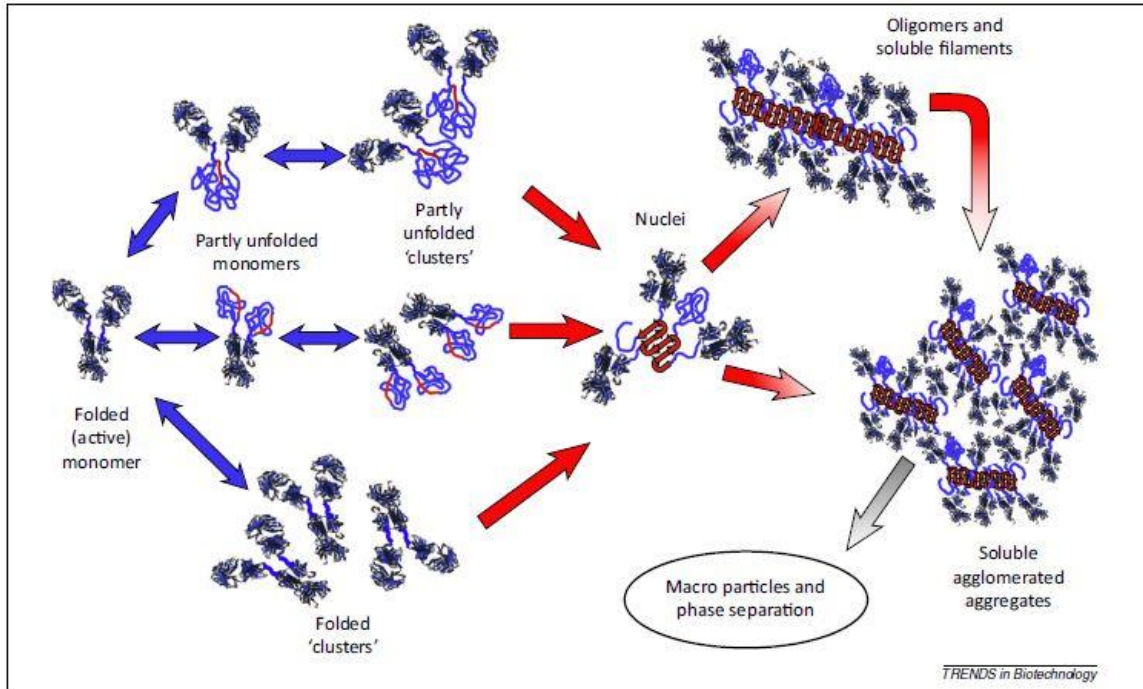
Figure 1. Aggregation pathways. Native and non-native pathways to aggregation, including progression to nucleation, soluble filaments and/or agglomerates, and precipitation. From "Therapeutic protein aggregation: mechanisms, design, and control" by C. J. Roberts, 2014, *Trends in Biotechnology, 32*(7), p. 373. Copyright 2014 by Elsevier Ltd.

Approaches to Monitor Aggregation

As the use of mAbs as drugs has gained momentum, the need to measure and monitor aggregation has increased with the need for versatile assays with high resolution. Aggregation monitoring assays can be split into two classes: direct aggregation monitoring, and indirect aggregation monitoring. Direct monitoring uses some characteristic of the aggregate to visualize it, but is only successful once the aggregation event has occurred. Meanwhile, indirect aggregation monitoring focuses on some aspect of an individual aggregation pathway, and visualizes the propensity of a mAb to follow that process.

Direct aggregation monitoring includes assays such as High Pressure Liquid Chromatography Size Exclusion Chromatography (HPLC-SEC), Dynamic Light Scattering (DLS), and Differential Static Light Scattering (DSLS). HPLC-SEC monitors the presence of protein particles separated by size by monitoring absorbance at 214 nm and/or 280 nm. Larger proteins flow through the column faster than smaller proteins and when compared to a set of molecular weight standards, size estimates can be made, including the presence of monomer and soluble aggregated mAb (Shi et al., 2013). DLS evaluates the scattering of light as it hits subvisible particles, allowing for a measurement of particle size (Fincke, Winter, Bunte, & Olbrich, 2014). Lastly, DSLS measures the scattering of light at 600 nm over a time period, which allows for the monitoring of aggregate formation (Goldberg et al., 2011). HPLC-SEC is limited in its capacity for number of samples processed in a reasonable amount of time, while DLS and DSLS can be used for high throughput screening. While these methods are adept at aggregation evaluation, there is a lack of clarity as to why or how those aggregates formed.

Indirect aggregation monitoring includes assays such as polyethylene glycol (PEG) and Ammonium Sulfate (AS) solubility, which reflect native aggregation propensity. Additionally, other techniques include circular dichroism (CD), differential scanning calorimetry (DSC), and differential scanning fluorescence (DSF), which reflect conformational stability, or propensity for non-native aggregation. PEG induces aggregation by an excluded volume effect, which encourages protein-protein interactions (Gibson et al., 2011). AS works in a similar manner as PEG, in which free water molecules are bound, thus dehydrating the protein's surface, and encouraging protein-protein interactions in the native state (Yamniuk et al., 2013). CD measures absorbance

of polarized light through which changes in conformational structure, as a function of heat, can be derived (Banks et al., 2012). DSC involves heating a reference cell and a protein containing cell; the difference in energy required to heat the cells is a function of protein concentration and temperature, and reflects the thermal stability of the protein (Johnson, 2013). DSF reports increase in environmental hydrophobicity as function of temperature, as a fluorescent dye binds the evaluated mAb and begins to fluoresce as hydrophobic core residues are exposed due to unfolding (Goldberg et al., 2011).

Both PEG and AS solubility have been developed into bench scale high throughput assays. CD and DSC are limited to low throughput capacity, while DSF shows high throughput capability. It is worth noting, however, that the assays that monitor native and non-native aggregation propensity do not tend to overlap, in that predictions of native aggregation do not generally make suggestions of non-native aggregation propensity (Alsenaidy et al., 2014; Yamniuk et al., 2013). The focus on high throughput screening is vital when it comes to aggregation propensity evaluation, both in the screening of development candidates from large panels of mAbs, as well in screening for formulation characteristics for a single mAb.

Stresses Encountered by Antibody Based Drugs

The production of a mAb based drug involves an extensive purification process, followed by filling, shipping, and long term storage. The process of generating a mAb based drug is fraught with various stresses, all of which can have a negative impact on the drug product if it is not stable. During the purification process mAbs are exposed to

low pH, as initial purification generally involves a low pH elution, and then extended

hold for viral inactivation (Vazquez-Rey & Lang, 2011). Upon purification, the mAb

undergoes a variety of processing steps including concentration, mixing, and filling

which can cause shearing and agitation stress. Finally, even shipment and storage can

expose a mAb based drug to stress, as most mAbs are frozen as bulk drug product prior to

filling and shipping, while the shipment process is generally performed with liquid

product, which may be exposed to variable temperatures (Vazquez-Rey et al., 2011).

While aggregated mAbs can be cleared during the purification process,

downstream processing related stresses can generate irreversible aggregates, if the mAb

is not stable. Multiple studies have been performed that monitor how some of these

stresses affect mAbs. Freeze/thaw cycling has reportedly produced native aggregates,

while heating generates non-native aggregates (Hawe et al., 2009). Agitation induced

stress produces non-native aggregates (Kiese, et al., 2008), while formulation and pH

variance can have both beneficial and detrimental effects on a mAb stability (Goldberg et

al., 2011; He et al., 2010; Yamniuk et al., 2014). While these studies have shown that

aggregation can be caused by assorted stresses, the use of a panel of stresses to delineate

a panel of mAbs for stability inference, or ranking, has not been reported.

Thermal Stability and Solubility are Indicative of Aggregation Propensity

It is widely accepted that protein aggregation, along the non-native pathway, is

caused by protein conformational instability, or partial unfolding of a protein. The

unfolding of the protein exposes the hydrophobic core residues, which then come

together with other partially unfolded monomers to form dimers, and ultimately act as a nucleation point for mass aggregation (Goldberg et al., 2011). Thus, conformational stability is indicative of a protein's propensity for non-native aggregation. Thermal stability is accepted as being a viable method to monitor a protein's conformational stability, through the determination of its melting point (Tm) or hydrophobic exposure temperature (Th) (He et al., 2010). The most established method for monitoring a protein's thermal stability, and characterize conformational stability, is DSC (He et al., 2010). Unfortunately DSC analysis is restricted by low throughput sample analysis, severely limiting the number of samples that can be processed in a reasonable amount of time. Fortunately, a relatively new technique has gained popularity: differential scanning fluorimetry (DSF). DSF allows for high throughput monitoring of conformational stability. Multiple studies have found highly correlative data between DSC and DSF, suggesting that DSF can successfully monitor protein thermal stability, and ultimately non-native aggregation propensity (Goldberg et al., 2011; He et al., 2010; Shi et al., 2013).

Differential scanning fluorescence uses a fluorescent dye, which is quenched in aqueous environments, but fluoresces under hydrophobic conditions. As such, under heat exposure, mAbs begin to lose conformational stability and unfold, exposing hydrophobic core residues, which can be measured as an increase in fluorescent signal (He et al., 2010). The benefits of DSF over DSC include low protein consumption, µg scale, and high throughput, 48-well up to 384-well plate measurement. However, DSF does have some drawbacks, namely an incompatibility with some commonly used excipients, such as surfactants, as the hydrophobic nature of those excipients masks the detectible signal

given off by the protein under standard working concentrations (Shi et al., 2013). While DSC does not suffer from this limitation, the inclusion of surfactants is only necessary for formulation screening, not stability mapping.

As previously discussed, native aggregation begins with the reversible association of monomers, based on uncharged surface hydrophobic patches. As the native state associations increase, the mAbs will ultimately form irreversible aggregates, generally through the formation of β-sheets with proximal mAbs. This raises the question of how to monitor native aggregation propensity, as the end result is an irreversible aggregate formed of misfolded monomers, which is indistinguishable from a non-native state aggregate, although attained by a different pathway (Roberts et al., 2014).

Ammonium sulfate (AS) is one of the strongest Hofmeister kosmotropes, is soluble at high concentrations, and is active from pH 2 – 10. AS precipitation is also known as "salting out" a protein, and has been used for decades as a method for protein fractionation and precipitation. The theory on its mechanism of action is that the strong polar sulfate anion binds water, thus dehydrating protein surfaces, and encouraging protein-protein interaction and precipitation. Most importantly for evaluating native aggregation is that because AS encourages hydrophobic interactions, it stabilizes the hydrophobic core, thus stabilizing the native conformation of the protein itself. This theory is supported by the fact that proteins that have been precipitated by AS can be re-solubilized by removal of the AS, thus returning nearly all material to its monomeric state (Yamniuk et al., 2013).

The establishment of the kosmotrope based solubility (KBS) assay as a means for evaluating native aggregation propensity comes with some qualifications. With respect to mAbs, the solubility range has been shown to be narrower than for other proteins, 1.2 – 1.6 M for mAbs as opposed to 0.7 – 2.0 M for small Adnectins. This was reportedly caused by sequence homology by mAbs, with only ~10% of the protein sequence comprised of the complementarity determining regions (CDRs) which will have construct to construct variability, while Adnectins had as much as 30% sequence variability (Yamniuk et al., 2013). However, even with the tighter range of solubility for mAbs, the KBS assay was able to reproducibly show solubility differences, while traditional methods, such as ultrafiltration and dynamic light scattering, were incapable of differentiating between mAb native aggregation propensity (Yamniuk et al., 2013).

Assessment of Stress Sensitivity to Rank the Stability of a Monoclonal Antibody Panel

Significant work has been done in studying aggregation of proteins, especially antibodies. However, in most situations those efforts tended to focus on a single aggregation pathway, or single stress, such as in the work by Yamniuk et al. (2013), Shi et al. (2013), and He et al. (2010). There have been efforts that focused more broadly on both aggregation pathways, such as the work by Banks et al. (2012), and Goldberg et al. (2011). These examples were more concentrated on efforts to identify the most stable formulation for a clinical development mAb. There is a need to study both aggregation pathways, jointly, as a process for defining and comparing the stability of a set of mAbs for selection as a development candidate. Overall, there has been little focus on

characterizing both aggregation pathways. This study focused on the propensity of mAbs for both aggregation pathways and aimed to establish a unified rationale for ranking of a panel of mAbs based on their aggregation propensities under different stress conditions, to identify top candidates for further development. This leads to greater insight into determining the worthiness of a mAb, from a stability perspective, for development.

Studies that focus on the non-native aggregation pathway define metrics for sample comparison. DSF defines Tm, which can be directly compared to other mAbs, or other formulations. However, this value does not reflect the propensity for native aggregation. The same is true for studies in native aggregation. KBS defines ASm, or the midpoint in AS solubility, which can also be used to rank proteins along their propensity for native aggregation. Another issue with both measurements is that there is no weight imposed on their values. For example, when comparing two mAbs, a 3 °C higher Tm as measured by DSF is difficult to classify on its own, or even if compared side-by-side with KBS results that shows a 0.2 M difference in ASm. How significant is a difference of 3 °C Tm, or 0.2 M ASm, when considering multiple mAbs as potential development candidates? Ultimately, their shortcoming is that the values do not speak to each other, and with a single evaluation the data may not be indicative of the robustness, or lack thereof, of a mAb. The general agreement is that stabilizing the biophysical properties of a mAb will identify a successful formulation (He et al., 2010). Taking that concept a step further is that the ability to comprehensively rank instabilities in the biophysical properties of a selection of mAbs will help to rank the best candidates for development.

This study utilized a series of stresses mimicking stresses a drug product would be exposed to, which aimed at exacerbating each aggregation pathway, with the goal of monitoring the differential stability, as measured by change in Tm and ASm, as a function of stress. The explicit intention was to derive a single metric unifying ASm and Tm, and use this value to rationally rank order a panel of mAbs for stability, in the event that ASm and Tm values do not rank similarly. This is not a formulation screening process but rather an empirical evaluation of multiple mAbs, taking into account both native and non-native aggregation propensities. Formulation will always play a role in the developmental success of a mAb. However, by identifying the most stable mAbs early on, the risk is reduced for those potentially investing significant resources into a mAb which cannot be stabilized through the addition of excipients (Lauer et al., 2012).

Chapter II

Materials and Methods

The following section details the materials and techniques used throughout the study. Briefly, mammalian cell culture was used to produce IgG, which was then purified by Protein A chromatography. A series of assays were then used to characterize the panel of mAbs in an unstressed state. Finally, the mAb panel was exposed to a series of stresses, and further evaluated for change in aggregation indicating values.

Production of Monoclonal Antibody Panel

A panel of mAbs was needed for this study. The selected mAbs, mAbs A – D, and F, are human IgG_1 variants, and were all expressed, purified, and processed under the same conditions. The production of these mAbs was done by transient transfection in HEK293F mammalian cells. The 293F cells were brought up in fresh FreeStyle^{TM} 293 expression medium (Life Technologies, Carlsbad, CA) to a density of 2×10^6 cells/mL. Transfection quantities reported are for 1 L cell culture transfections. Plasmid DNA of the mAb heavy chain and light chain were mixed at 750 µg each into 53.75 mL 150 mM NaCl, then 6 mL of polyethylenimine is added. The plasmid solution was mixed gently by pipetting, and incubated for 10 minutes at room temperature. The plasmid solution

was then added to the 1 L cell culture, while swirling the cells gently. Transfected cells were placed in a 37 °C incubator with 8% CO_2 on a shaking platform at 40 rpm.

After seven days of expression, the media containing the secreted mAbs, was clarified by centrifugation and vacuum filtration. The cell culture media was centrifuged at 1500 x g at 4 °C for 10 minutes in a Sorvall® Legend RT swing bucket centrifuge, to pellet the suspension cells. The supernatant was carefully decanted, and filtered through a 0.22 μm polyethersulfone membrane Nalgene vacuum filter. The clarified supernatant containing the expressed mAbs was then stored at 4 °C until purification.

The mAbs were purified by Protein A based affinity chromatography on a liquid handling system. An ÄKTApurifier 10 (GE Life Sciences, Little Chalfont, Buckinghamshire, UK) fast protein liquid chromatography system (FPLC) was used to automate the purification of the mAbs. All purifications were done at 4 °C and 1 mL/min flowrate. A 1 mL MabSelect SuRe® protein A column from GE Life Sciences was equilibrated with 8 mL of 1x PBS (7 mM Phosphate, 154 mM NaCl pH 7.4, Lonza, Walkersville, MD), plus 0.05% Sodium Azide (PBSN). The cell culture media was then loaded over the column, immobilizing the IgG on the affinity resin. The mAb depleted column flow through was collected for repeat purification if the initial process failed to immobilize all of the IgG present in the solution. The protein A column was then re-equilibrated with 10 mL of PBSN, and the IgG was eluted with 12 mL 100 mM Glycine-HCl pH 2.5. The elution was collected in fractions, with pooling defined by the 280nm absorbance chromatogram. The eluted mAb was neutralized by the addition of 1:10 dilution of 1.0 M Tris-base, 1.0 M NaCl pH 8.5.

Upon purification, from cell culture supernatant, the mAbs were then buffer exchanged into 1x PBS (Lonza) by ultrafiltration/diafiltration, (UF/DF) with an Amicon® 15 mL, 30 kDa molecular weight cutoff centrifugal filter (EMD Millipore, Billerica, MA), at 4000 x g, 4 °C. Through compounding dilution, the mAbs were all UF/DF processed to a dilution of $\geq$ 1:500 into 1x PBS, and a final concentration of 25 ± 1 mg/mL. The panel of mAbs was quantified by absorbance at 280 nm with a NanoDrop 2000 spectrophotometer, with calculated theoretical extinction coefficients (Thermo Scientific, Rockland, MD). The mAbs were all 0.22 μm syringe filter sterilized, and stored at 4 °C for the duration of this study.

Assays Used to Assess Monoclonal Antibody Panel

Differential scanning fluorescence was a key assay used to determine the Tm of the panel of mAbs under neutral and stressed conditions. The protocol for the DSF evaluation was based on the reported work of Goldberg et al. (2011) and He et al. (2010). Samples to be tested were diluted to 1.0 ± 0.1 mg/mL in the buffer matching present mAb formulation. Each sample was tested in duplicate with 15 μL of dilute mAb in each well, for a total of 30 μL per sample or approximately 30 μg per sample per assay. SYPRO® Orange dye was purchased from Life Technologies (Carlsbad, CA) as a concentrate in dimethyl sulfoxide, and used at a final dilution of 1:1000 in the assay. At this dilution, the dye was reported to not induce any changes in the thermal stability of mAbs (Goldberg et al., 2011). The dye was thawed by incubation in a heat block at 37 °C for 3-5 minutes, and then diluted 1:500, in buffer matching the mAb formulation to be

tested, by adding 2 µL of dye to 998 µL of appropriate buffer. The diluted dye solution and diluted mAbs were mixed at a ratio of 1:1, and loaded onto a 48-well PCR plate, 30 µL solution per well. The mAb and dye solution was mixed immediately prior to starting the assay. Matched blanks were also included on the plate, prepared by mixing 15 µL of formulation buffer 1:1 with dye prepared in that same buffer. Blanks were run $\geq$ 2 wells per formulation per plate. Plates were sealed with MicroAmp[TM] optical adhesive film (Applied Biosystems®, Carlsbad, CA).

Thermal cycling and fluorescence measurement of the DSF assay was performed with a StepOne[TM] Real-Time PCR system (Applied Biosystems®, Carlsbad, CA). Fluorescence was measured with the ROX reporter system. The samples were exposed to a temperature range from 25 – 99.9 °C, holding at set temperature for 1 minute, reducing the temperature by 1 °C for 10 seconds and measuring the fluorescent output of the plate, and ramping up 2 °C. All temperature changes were set at max speed (100% ramp speed).

The DSF data output was taken from the raw data, red emission channel. Samples were matched with blanks in corresponding formulation buffer, and a direct subtraction of the averaged blank was taken for each corresponding cycle. The blank subtracted data was then plotted on an X-Y scatter plot. The point of maximum fluorescence was marked as the point of total unfolding, prior to aggregation and the quenching of the fluorescent signal. Data beyond this peak fluorescent signal was excluded from the data analysis. A Boltzmann sigmoidal curve (Eq.1) was fit to the data using GraphPad Prism® software, where A is the bottom saturation, B is the top saturation, and Tm is the midpoint of the sigmoidal curve, or the melting point of the mAb.

Equation 1.
$$Y = A + \frac{B - A}{1 + e^{(Tm - X)/slope}}$$

Kosmotrope based solubility was the other primary assay used in this study; it was used to determine the ASm of the panel of mAbs under neutral and stressed conditions. The protocol for the KBS assay was based on the reported work by Yamniuk et al. (2013). The assay is composed of mixing 30% final volume dilute mAb solution with varying concentrations of 70% final volume AS. Based on the solubility of the mAb, precipitation will occur. The precipitation event is rapid and stable, with no observed change in concentration seen over the course of several hours. Samples to be tested were diluted to 1.5 ± 0.1 mg/mL in the buffer matching tested mAb formulation, for a final concentration of 0.45 mg/mL upon dilution in the assay. Each sample was tested in duplicate with 10 concentrations of ammonium sulfate (AS). The final concentration of AS used in the assay is 0 M, and from 0.5 M – 2.0 M in 0.25 M increments, with two additional 0.125 M increment data points added in the "salting-out" region. These 0.125 M concentrations were identified by a low resolution pre-test, described below. The 0.5 M lower concentration was selected because all mAbs in the panel showed full solubility by 0.5 M, but also because at lower concentrations, generally below 0.3 M AS, solubility is actually improved through electrostatic interactions (Yamniuk et al., 2013). The 2.0 M upper concentration was selected because the mAb panel showed no solubility at that point or beyond.

Due to the steep slope of solubility, variability in the assay can stem from a lack of data points along the partial solubility portion of the curve. To predict the point of

solubility, a 4 dilution rapid test was designed to use minimal protein, while identifying

approximately where in the solubility curve those additional points would be beneficial.

In 4 PCR tubes, 10 μL of dilute mAb was added to 23.3 μL of AS in 0.25 M increments.

The exact range of AS used was matched to the known ASm values of the unstressed

mAb. For instance, mAb A had a calculated ASm of 1.28 M; thus the rapid test used

concentrations of 0.75 – 1.50 M AS to evaluate the stressed material for changes in

solubility, and the appropriate AS concentrations to be used in the full assay. The mixed

solution was allowed to sit for 5 minutes, then the tubes were centrifuged for 30 seconds,

and the presence of pelleted precipitate was used to identify points of insolubility. The

lowest concentration of AS with visible precipitate was identified, and concentration

points of 0.125 M were added above and below that concentration. For example, if

stressed mAb A was evaluated with 0.75 – 1.50 M AS, and the tube with 1.25 M AS was

the lowest concentration with a visible pellet, then 1.125 M and 1.375 M AS points were

added to the testing scheme for mAb A. A sample plate layout can be seen in Figure 2.

Each sample received a custom set of two additional AS concentrations, in addition to the

standard set noted above.

	1	2	3	4	5	6	7	8	9	10	[AS]-blank	
mAb	2.00	1.75	1.50	**1.38**	1.25	**1.13**	1.00	0.75	0.50	0	2.00	0.88
A	2.00	1.75	1.50	**1.38**	1.25	**1.13**	1.00	0.75	0.50	0	1.75	0.75
mAb	2.00	1.75	**1.63**	1.50	**1.38**	1.25	1.00	0.75	0.50	0	1.63	0.50
B	2.00	1.75	**1.63**	1.50	**1.38**	1.25	1.00	0.75	0.50	0	1.50	0
mAb	2.00	1.75	1.50	1.25	**1.13**	1.00	**0.88**	0.75	0.50	0	1.38	
C	2.00	1.75	1.50	1.25	**1.13**	1.00	**0.88**	0.75	0.50	0	1.25	
mAb	2.00	1.75	**1.63**	1.50	**1.38**	1.25	1.00	0.75	0.50	0	1.13	
D	2.00	1.75	**1.63**	1.50	**1.38**	1.25	1.00	0.75	0.50	0	1.00	

Figure 2. Sample plate layout for KBS assay. Columns 1 – 10 list tested concentration of AS in M, concentrations in bold are custom points added to increase data points on partial solubility curve, [AS]-blank are wells with no protein, to be used as absorbance blanks for matched [AS] wells

Once the set of AS concentrations to be tested was set by the rapid screen, the full assay was run. In a 96-well U-bottom plate, stock concentrations of AS were loaded into the well at 70% of the final volume, and dilute mAb was added to the AS concentrations at 30% final volume, for a final total protein concentration of 0.45 mg/mL. The plate was mixed for 30 seconds using the plate mixing feature of the SpectraMax M2e plate reader (Molecular Devices, Sunnyvale, CA), then incubated static at room temperature for 10 minutes. After the incubation, the plate was then centrifuged at 2000 x g for 5 minutes at room temperature, to pellet precipitated protein. The assay was measured by quantifying the residual soluble protein in each well. To quantify, 2 µL of solution was measured by absorbance at 280 nm on a NanoDrop 2000, blanked with a matched [AS]-formulation buffer blank. Care was taken to not disturb the pelleted precipitate in the well, when pipetting the sample for quantification.

Two versions of the KBS assay were used, a "full volume" version, and a "half volume" variant. The full volume assay used 70 µL of AS and 30 µL of dilute mAb per well, while the half volume assay used 35 µL of AS and 15 µL of dilute mAb per well. There did not appear to be any direct benefit to the assay in using the full volume, with the exception of ease of sample quantification during the reading step of the assay, as decanting 2 µL from 100 µL without disturbing the precipitation pellet was slightly easier than decanting 2 µL from 50 µL. The full volume assay required 900 µg of sample, while the half volume reduced sample need to 450 µg. A comparison of the full volume versus half volume KBS assay with mAb A showed a difference in ASm of only 1.5% (Figure 3).
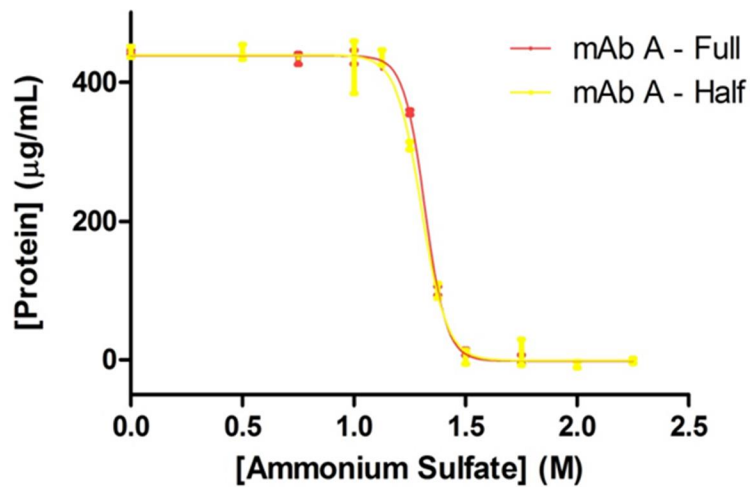

Figure 3. Solubility curve comparison of full and half volume assays. KBS assays show superimposable plots, and a ASm variance of 1.32 M for the full volume and 1.30 M for the half volume assay, or only 1.5% difference

Once the KBS plate was read, the data was then processed, first by converting the measured A280 into protein concentration by application of the appropriate extinction coefficienct to each mAb's data set. Then, the data was plotted on an X-Y scatter plot graph and a sigmoidal dose response curve was fit to the data (Eq 2) using GraphPad Prism® software, where A is bottom saturation, B is top saturation, and ASm is midpoint in the sigmoidal curve, or the equilibrium solubility point of the mAb.

Equation 2.
$$Y = A + \frac{B - A}{1 + 10^{(LogASm - X) * slope}}$$

Analytical HPLC-SEC was used to determine purity of the purified mAb panel prior to application of stress. The HPLC-SEC analysis was performed on an Agilent 1100 liquid chromatography system. For mAbs A, B, and D, 1 mg/mL dilutions were prepared in 1x PBS, and 20 μL was injected onto a Phenomenex® BioSep-SEC-s3000 300 x 7.8 mm column (Torrance, CA). The column was run with an isocratic elution, composed of 1x PBS in the mobile phase, a flowrate of 1 mL/min, and a run time of 25 minutes. Absorbance at 280 nm was monitored. For mAb C, an alternate protocol was used, as mAb C appears to have adsorption issues with the BioSep-SEC-s3000 column. For mAb C, a 1 mg/mL dilution was prepared in 1x PBS, and 20 μL was injected onto a Phenomenex® PolySep-GFC-P 4000 300 x 7.8 mm column. The column was run with an isocratic elution, composed of 100 mM Phosphate, 125 mM Arginine, pH 10.8 in the mobile phase, a flowrate of 0.25 mL/min, and a run time of 50 minutes. Absorbance at

280 nm was monitored. All samples were 0.22 μm filtered prior to application to the chromatography column.

Stresses to the Monoclonal Antibody Panel

Four stresses were applied to the mAb panel to determine relative sensitivity as a function of native, non-native, and overall aggregation. The stresses were elevated isothermal incubation, agitation, freeze-thaw cycling, and solution pH. Stressed samples were evaluated on NanoDrop for change in soluble protein concentration, by DFS for change in Tm, and KBS for change in ASm, as compared to the unstressed control. All tests were performed as previously described, and executed within an hour of cessation of stress. Any remaining sample was stored at 4 °C.

To incur elevated isothermal stress, the mAb panel was incubated at 45 °C, which is ≥ 20 °C below the calculated Tm of all mAbs in the panel to prevent full denaturation, but rather to apply a constant stress to the secondary structure of the mAbs, as recommended by Yamniuk et al. (2013). The mAb panel was prepared for incubation by generating a 1 mL sample at 3 mg/mL, dilutions were performed in 1x PBS, samples aliquots were kept in 1.5 mL microfuge tubes and stored static in the dark for the duration of the incubation. After 1 week, all samples were inspected visually for presence of precipitate, and 0.5 mL of each sample was removed from the incubator, while the remaining material was incubated for an additional 1 week at 45 °C. Both week 1 and week 2 samples were tested immediately upon removal from incubator.

The agitation induced stress protocol was based on the work reported by Kiese et al. (2008), in which agitation was reported to trigger mAb aggregation and precipitation in solution, in a manner believed to be caused by unfolding due to the interaction of the mAbs at the air-gas phase. For the experiment, 1 mL at 5 mg/mL of each mAb was prepared, and stored in 2 mL silanized glass vials (Supleco, Bellefonte, PA) sealed with Parafilm®. The vials were stored vertically in an opaque box, at room temperature, on a shaking platform (VWR Shaker model 3500, Radnor, PA) set at 200 rpm. Visual inspection and 200 μL samples were taken at specific time points: 1, 3, 7, and 14 days. Kiese et al. (2008) reported that headspace, or the volume of liquid with respect to the capacity of the vial did not affect the results. Thus the change in volume during agitation time points was not expected to affect the outcome of the experiment. Each time point was tested immediately upon removal from agitation.

The freeze thaw cycling protocol was based on the work of Hawe et al. (2009), who reported the formation of aggregates that were formed of native structure mAbs, suggesting that freeze-thawing triggers the native aggregation pathway, although they did not suggest a mechanism of action. For the experiment, 1 mL at 3 mg/mL of each of the mAb was prepared, in 1x PBS, and stored in a 1.5 mL microfuge tube. The samples were frozen by storing at -80 °C for ≥ 15 minutes, followed by thawing in a 25 °C heat block for 20 minutes. The vials were inspected visually for the presence of precipitate, 0.5 mL of each sample was taken after 5 freeze-thaw cycles, while the remainder was exposed to an additional 5 cycles, for a total of 10 freeze-thaw cycles. After 5 and 10 cycles, samples were tested immediately upon thawing.

Solution pH was selected as Yamniuk et al. (2013) reported variation in the ASm of several mAbs, as a function of pH, while specific buffering agent did not seem to have any effect on the results. Four pH controlled buffer solutions were prepared to assess the effects of pH on aggregation propensity, the solutions all contained 50 mM buffering agent, and were 0.22 μm vacuum filtered prior to use. The solutions were: Citric Acid – Sodium Citrate pH 4.0, 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.5, Bis-Tris Propane pH 7.0, and Tris-Base pH 8.5. To adjust the pH of the mAbs, 60 mL of the concentrated mAb stock solution was added to 540 mL of the pH control solution, for a final concentration of 2.5 mg/mL. The mAb stock formulation of 1x PBS was verified to minimally affect the pH adjusted solution. Thus the spiked mAb panel was successfully exposed to the stress pH through dilution. Upon exposure to the pH controlled solutions, the samples were mixed by vortexing, and incubated at room temperature for 10 minutes. Samples were inspected visually for the presence of precipitate before analysis. In the KBS assay, the AS concentrations were in matched buffer and pH for ASm evaluation.

Chapter III

Results

The focus of this study was to understand the conformational and colloidal stability contribution to aggregation propensity for a monoclonal antibody. To do this, a panel of four mAbs were generated and characterized, stresses were then applied and further stability characterization was completed. The mAbs that comprise the panel are all human IgG1 mAbs, and conform to a baseline set of expression and stability criteria to enable further stability studies. The mAbs were then subjected to a series of four stresses, each stress selected because it mimics a potential stress a mAb drug would face during manufacturing and storage, coupled with reported aggregation pathway specificity. The effect of each stress was monitored as a function of change in colloidal and conformational stability, as well as formation of insoluble aggregate. Through evaluation of the sensitivities to these stresses, understanding of the stability of each mAb in the panel was gained, and used to rank the mAb panel by overall stability.

The Monoclonal Antibody Panel

Aggregation propensity in mAbs is generally dictated by the Fab domain of an antibody (Yamniuk et al., 2013). Thus, to study the effects of various stresses on mAb aggregation, it is necessary to have a panel of mAbs with highly heterogeneous Fab

domains, as high homology could reduce the diversity of response to stress. The mAbs

selected for the panel all have human IgG_1 Fc domains, but diverse variable regions in

both the heavy (V_H) and light (V_L) chain. The sequence diversity was evaluated by

comparing the homology of the V_H and V_L using the NCBI Protein Blast Tool (Boratyn

et al., 2013) (Table 1). There is significant diversity in the V_H and V_L sequence, except

for some similarity between mAbs C and F. Initially, five mAbs were selected for

evaluation. Four of the mAbs, mAbs A – D, had acceptable expression rates of greater

than 25 mg/L, and were capable of attaining the stock concentration of 25 ± 1 mg/mL.

One mAb failed the cutoff for expression rate, mAb F, which expressed at 5.4 mg/L.

Additionally, mAb F appeared to partially clog the filter membrane during the UF/DF

processing, resulting in extended centrifugation times for concentration, in comparison to

the other mAbs on the panel, thus making it difficult to attain a concentration of 25

mg/mL. As such, mAb F appeared to show characteristics of a poor developability

profile. Thus, mAb F was removed from the final panel, but with the available material,

unstressed DSF and KBS evaluation was performed.

Table 1.

Sequence Homology of V_H and V_L of Monoclonal Antibody Panel

Sample	mAb A	mAb B	mAb C	mAb D	mAb F
mAb A	-	71% (84%)	68% (81%)	50% (68%)	67% (81%)
mAb B	71% (84%)	-	61% (85%)	61% (68%)	62% (84%)
mAb C	68% (81%)	61% (85%)	-	44% (64%)	96% (99%)
mAb D	50% (68%)	61% (68%)	44% (64%)	-	43% (64%)
mAb F	67% (81%)	62% (84%)	96% (99%)	43% (64%)	-

Note. Sequence homology reported as % homology as $V_H (V_L)$, generated with the NCBI Protein Blast Tool

Characterization of Antibody Panel

Monoclonal antibodies A to F were recombinantly expressed in mammalian cells and purified using a single step Protein A purification followed by UF/DF buffer exchange and concentration. The generated panel of mAbs was evaluated by HPLC-SEC for monomeric purity. Purity is important, as significant amounts of impurities or degradation products could potentially skew assay results. Based on HPLC-SEC, the unstressed mAbs all had acceptable levels of monomeric purity, with mAb B having the lowest purity at 93.5%, and mAbs A, C, and D having >98% purity (Figure 4).

The basis of this study is to evaluate the effects of stresses to a mAb panel as a function of change in ASm and Tm values, as indicative of aggregation propensity along the native and non-native aggregation pathways, with the intent to rank the mAbs by order of total stability. To determine change caused by stress, baseline unstressed ASm values were first established. To accommodate all samples on a single 96-well plate, to avoid plate-to-plate variability, unstressed material was not run in parallel with each stressed sample while running the KBS assay. Rather, the unstressed panel was evaluated once by performing the KBS assay in octuplicate for each mAb and solubility curves for each sample were obtained (Figure 5a), and all other solubility curves for stressed samples were also generated. Based on unstressed ASm values, ranking lowest solubility to highest, were mAbs C, A, D, and B, ranging from 1.05 – 1.50 M.
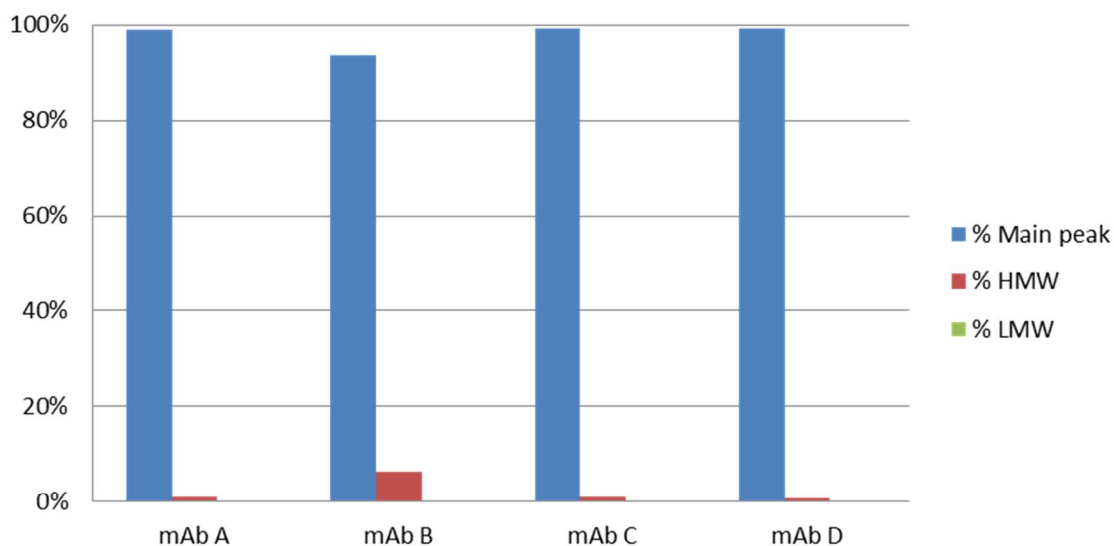
Figure 4. HPLC-SEC purity evaluation of mAb panel. Main peak represents monomer, high molecular weight (HMW) represents oligomers, and LMW represent degradation products. None of the samples have LMW species, mAb B contains 6.5% HMW species, while the remaining mAbs contain less than 1.5% HMW species
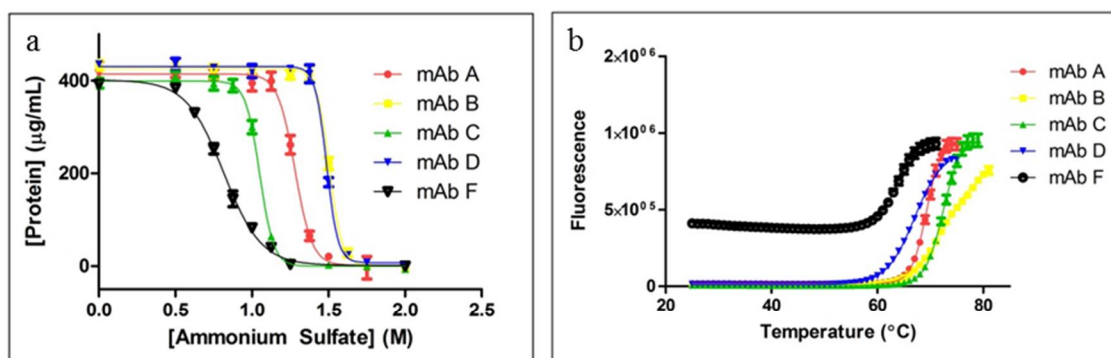
Figure 5. Sample curves for the unstressed mAb panel. (a) KBS solubility curves, error bars represent standard deviation, n = 8 for mAbs A – D, mAb F n = 2, connecting line is best fit sigmoidal dose response curve. (b) DSF thermal stability curves, error bars represent standard deviation, n = 2, connecting line is best fit Boltzmann sigmoidal curve. The baseline saturation of mAb F is 35-fold higher than the mAbs A – D.

To determine unstressed Tm values, each mAb was evaluated by the DSF assay $\geq$ 8 times. Unlike the KBS assay, DSF required minimal amounts of material, and minimal additional effort, and the assay plate did not have space issues. Thus, the unstressed mAbs were included in parallel with stressed material in all DSF assays. The unstressed Tm values were defined by averaging all DSF measurements taken with unstressed material, sample thermal melt curves were obtained (Figure 5b), and all other thermal stability curves for stressed samples were also generated. Based on unstressed Tm values, ranking lowest thermal stability to highest, were mAbs D, A, C, and B, ranging from 67.0 – 73.0 °C.

A summary of the established baseline values for mAbs A – D can be found in Table 2. While differences in the Fab do not necessarily dictate variance in the ASm or Tm of a molecule, interestingly, all four of the mAbs on the panel have a unique ASm – Tm profile (Figure 6). As a point of comparison, the available mAb F material was used to generate baseline ASm and Tm values. Surprisingly, despite the high sequence homology between mAbs C and F, their stability profiles varied significantly, further highlighting how even minor changes in the Fab can alter stability of a mAb. The interest in mAb F lies in its apparent inability to express at an acceptable level, and noted issues with concentration by ultrafiltration, suggesting likely problems with development. As such, mAb F was evaluated by DSF and KBS to compare to the full mAb panel that was capable of expression and concentration requirements. The ASm calculated for mAb F was 0.82 M, and as seen in the solubility curve (Figure 5a), attained full solubility only at 0 and 0.5 M points. The Tm calculated for mAb F was 63.7 °C, and has a baseline fluorescence that was over 35-fold higher than the rest of the mAb panel (Figure 5b). As

shown in Figure 6, there is a clear separation in the stability indicating values attained for all five mAbs evaluated, with mAb F clearly having the lowest stability profile, as it has both the lowest ASm and Tm values. In contrast to mAb F, mAb B clearly had the top stability profile, with the highest ASm and Tm values. Ranking mAbs, A, C, and D based on their stability was more challenging given that their stability profile ranges from high solubility and low thermal stability (mAb D), low solubility and high thermal stability (mAb C), to mid-level solubility and thermal stability (mAb C). To discriminate the relative stability of the mAbs, the mAbs were subjected to a battery of tests.

Table 2.

Established Values for the Unstressed Monoclonal Antibody Panel

Sample	Stock Concentration	Monomeric Purity	ASm (M)	Tm (°C)
mAb A	25.46 mg/mL	98.9%	1.28	69.3
mAb B	26.00 mg/mL	93.5%	1.50	73.0
mAb C	25.49 mg/mL	99.1%	1.05	72.2
mAb D	25.37 mg/mL	99.2%	1.49	67.0

Note. Tm = Melting temperature, ASm = Ammonium sulfate solubility

Effects of Stress on Antibody Panel

A series of stresses were applied to the panel of mAbs to mimic production and storage related stresses that a mAb based drug is subjected to during manufacturing, shipping, and storage. Elevated isothermal incubation is commonly used as a method to increase the rate of degradation that would normally be seen in real-time over the course

of months to years. Agitation induced stress mimics stress that a drug would experience during transportation. Both elevated isothermal incubation and agitation induced stress have been reported to generate non-native conformation based mAb aggregates by Hawe et al. (2009) and Kiese et al. (2008). Additionally, the panel was subjected to freeze/thaw cycling, which drugs may be exposed to during storage, as well as solution pH induced stress, which mAbs are subjected to during initial production and final formulation. Freeze/thaw cycling was reported by Hawe et al. (2009) to result in native conformation aggregates, while Yamniuk et al. (2013) reported variance in ASm values as a function of solution pH, regardless of buffering agent. The intent of these experiments was to attempt to parse out relative sensitivities to stresses that a drug would be subjected to, while monitoring insoluble aggregate formation, colloidal stability, and conformational stability, as a method for interpreting overall stability.

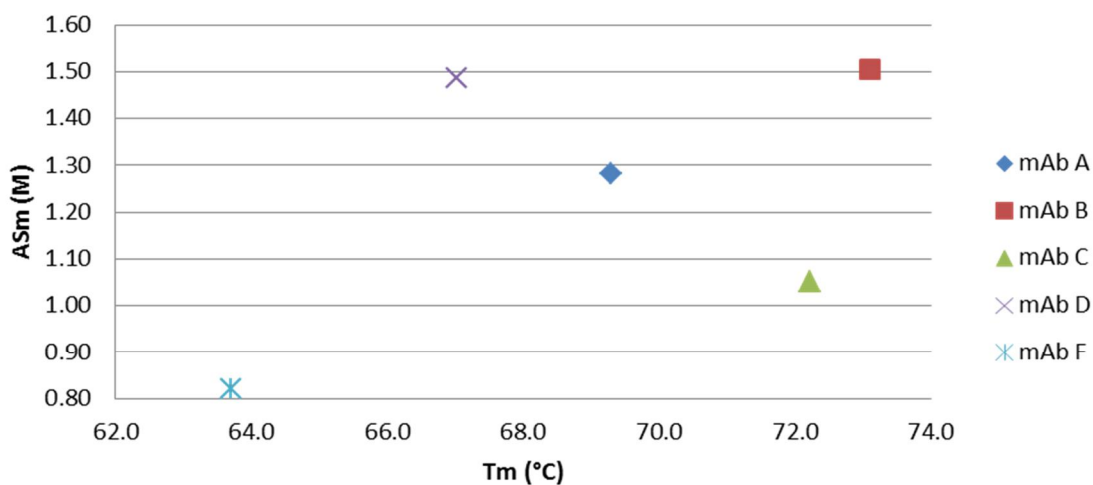*Figure 6.* Tm and ASm scatterplot of unstressed mAb panel. Points depict the average ASm and Tm values (n ≥ 8), calculated for each unstressed mAb. MAb F was included in the scatterplot (n = 2) to visualize separation of aggregation propensity characteristics between the panel, and mAb F which failed the expression and processing cutoff.

Elevated Isothermal Incubation

The panel of mAbs was subjected to elevated isothermal incubation, at 45 °C for 2 weeks. After incubation at 45 °C for 1 week, mAb C had small uniform sized particulates, while the rest of the panel had no evidence of insoluble aggregates. The measured soluble protein concentration for all samples was unchanged. After 2 weeks of incubation at 45 °C, all samples showed mild levels of precipitation. Measured soluble protein concentration showed a minor reduction for all samples, of < 0.05 mg/mL.

The stressed samples were evaluated by DSF and KBS after both time points (Table 3). After 1 week incubation the ASm values of mAbs A, C, and D were all lower than unstressed material, by between 0.03 – 0.08 M, while the Tm values were unchanged. This variance in ASm is surprising, as elevated isothermal stress was reported to affect non-native aggregation, which is monitored through change in Tm. Evaluation of samples after 2 weeks revealed a less substantial change in ASm, as mAbs A and C had a minor reduction of 0.01 M, as compared to unstressed, and mAbs B and D were unchanged. The Tm variations seen after 2 weeks were minimal, with the exception of mAb A showing a 0.5 °C increase. Based on the data from 1 week incubation, the ASm values appear to be reduced in mAbs A, C, and D. However, due to the subsequent week 2 time point results, it appears that the reduction in ASm may be due to assay variation, as the trending does not support a reduction in ASm as a function of elevated isothermal incubation.

Table 3.

Effects of Elevated Isothermal Incubation on Monoclonal Antibody Panel

	Unstressed		Day 7			Day 14		
Sample	ASm (M)	Tm (°C)	ASm (M)	Tm (°C)	Precip.	ASm (M)	Tm (°C)	Precip.
mAb A	1.28	69.3	1.25	69.4	-	1.27	69.8	+
mAb B	1.50	73.1	1.50	73.5	-	1.50	73.3	+
mAb C	1.05	72.2	0.97	72.2	+	1.04	72.3	+
mAb D	1.49	67.0	1.46	67.1	-	1.49	67.0	+

Note. ASm = Ammonium sulfate solubility, Tm = Melting temperature, Precip.= Observed precipitate, rated on quantity and size of particulate: - = none seen, + = minor quantity/small.

In an effort to ensure that heat stress can affect the ASm and Tm values of the mAbs a subset of the panel, mAbs A and D, were diluted to 2 mg/mL in PBS at 0.5 mL total volume, and heated to 60 °C for 15 hours in a thermal cycler. The 60 °C incubation temperature was selected as the highest temperature to affect thermal stability without fully thermally denaturing the mAbs, as it was 7 – 9 °C below Tm. This extreme heat stress generated heavy precipitation in both samples, with substantial changes in soluble protein concentration, suggesting that the increased temperature successfully stressed the mAbs. The soluble protein concentration, post clarification by filtration, for mAb A was 0.84 mg/mL (58% loss), and mAb D was almost a complete loss at 0.09 mg/mL remaining (96% loss). The loss of protein in mAb D made it unusable in DFS and KBS assays, mAb A was further evaluated by DFS and KBS and compared to unstressed material. The solubility evaluation showed only minor variations from the control, with a 0.03 M increase in ASm (Figure 7a). In the resulting DSF evaluation, the Tm value only shifts lightly with an apparent increase of 0.5 °C. This increase in Tm was caused by an increase in the ambient hydrophobic atmosphere, as can be seen by a greater than 5-fold

increase in baseline fluorescence (Figure 7b).   These results suggest that even extreme

heat stress only provides a transient stress to the mAb, which does not translate to an
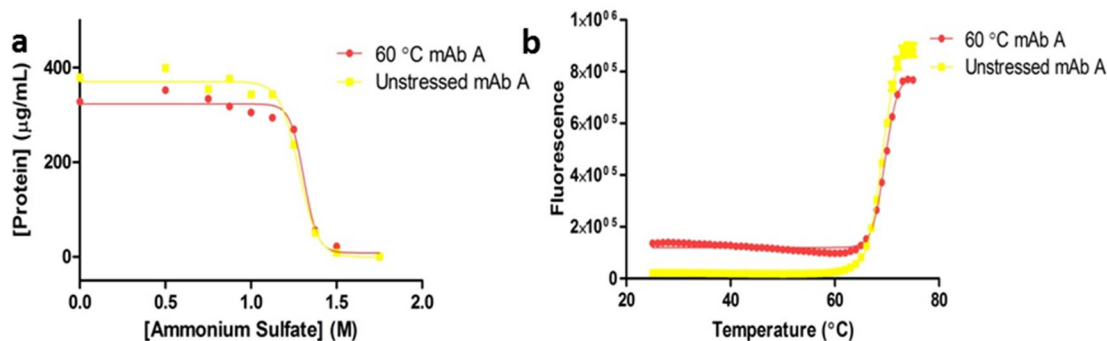
overall shift in Tm or ASm.

Figure 7. DSF and KBS evaluation of mAb subjected to extreme heat stress. MAb A
was heat stressed at 60 °C for 15 hours, and evaluated by KBS and DFS. (a) The KBS
assay shows very minor differences from the unstressed material, with a slightly lower
maximum concentration, which could be caused by slightly lower starting
concentration. (b) The Tm value of the super stressed material are minimally different
from the control, however there is a 5-fold increase in the baseline fluorescence,
suggesting an increase in the ambient hydrophobic environment.

Agitation Induced Stress

Agitation stressed samples were evaluated on days 1, 3, 7, and 14. Visual

observation of insoluble aggregate is summarized in Table 4. Again, the presence of

precipitate did not have much impact on the soluble protein concentration. The stressed

samples were evaluated by DSF and KBS and showed minimal difference from the

unstressed control (Figure 8). After 1 day of agitation stress, mAb A showed a 0.04 M

reduction in ASm, mAb C showed a 0.09 M reduction in ASm, while mAbs B and D

showed < 0.02 M reduction in ASm. However, it was noted that the remaining time

points (days 3, 7, and 14) did not share this trend in ASm reduction. Based on the data

set as a whole, the day 1 reduction in ASm does trend with the remaining data points.

The differences was thought to be a result of variability and not necessarily a function of

the agitation induced stress. Thus, overall agitation induced stress did not appear to have

an impact on the ASm or Tm values of the mAb panel.

Table 4.

Precipitate Observation After Agitation Stress of Sample

	Observed Precipitation After Agitation Incubation			
Sample	Day 1	Day 3	Day 7	Day 14
mAb A	+/-	+	+	++
mAb B	+	++	++	++
mAb C	+	++	++	++
mAb D	+/-	+	+	++

Note. Observed precipitate is rated on quantity and size of particulate: +/- = minor/hard to see, + = minor quantity/small, ++ = medium quantity/larger flakes
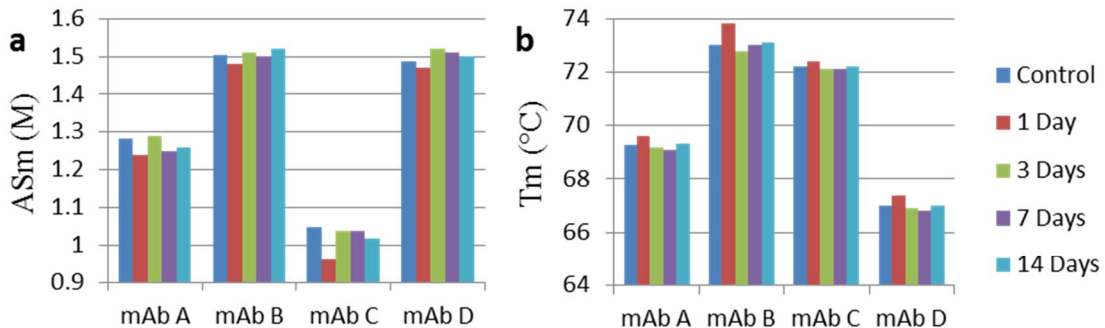

Figure 8. KBS and DSF evaluation of effects of agitation induced stress. (a) Agitation stress exhibited minor impact on ASm, with slight reduction in ASm from panel on day 1, assay variation cannot be ruled out. (b) No variation seen in Tm values, no trending as a function of time in stress can be seen between either KBS or DSF assay results.

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Freeze/Thaw Cycling

The panel of mAbs was subjected to freeze/thaw cycling ten times. KBS and DSF assays were performed on the samples after five and ten cycles. Visual observations of insoluble aggregate were made after each thaw cycle, and change in soluble protein concentration is reported (Table 5). After five freeze/thaw cycles, there was minimal change in soluble protein concentration, but after ten cycles, mAb A showed a 0.17 mg/mL reduction in concentration, while mAbs B – D showed between 0.03 – 0.06 mg/mL reduction in concentration. After five freeze/thaw cycles, no change was seen in either ASm or Tm with any of the mAbs in the panel. After ten freeze/thaw cycles no change was seen in Tm, but a minor reduction in ASm was seen in mAbs A and C, 0.03 and 0.06 M respectively, while mAbs B and D showed no change (Figure 9). Due to sample constraints, further freeze/thaw cycling was not performed, and because of the lack of additional cycle points to confirm a downward trend, assay variation cannot be ruled out. As such, it could not be conclusively stated that ASm and Tm values were impacted by freeze/thaw induced stress for these mAbs. However, the mAbs did appear to be impacted by this stress, as revealed by the observed visual precipitate and the reduction in soluble protein.

Table 5.

Precipitation and Concentration Change as a Function of Freeze/Thaw Cycling

	Precipitate Observation					Δ Conc (mg/mL)	Precipitate Observation					Δ Conc (mg/mL)
Sample	1x	2x	3x	4x	5x	5x	6x	5x	8x	9x	10x	10x
mAb A	-	+/-	+/-	+	+	0	+	+	+	+	+	-0.17
mAb B	-	+/-	+/-	+	+	-0.01	+	+	+	+	+	-0.06
mAb C	-	-	-	+/-	+	-0.01	+	+	+	+	+	-0.03
mAb D	-	-	+/-	+	+	0	+	+	+	+	+	-0.05

Note. Observed precipitate is ranked on quantity and size of particulate: - = none seen, +/- = minor/hard to see, + = minor quantity/small. Concentration change based on change from pre-stressed dilute sample
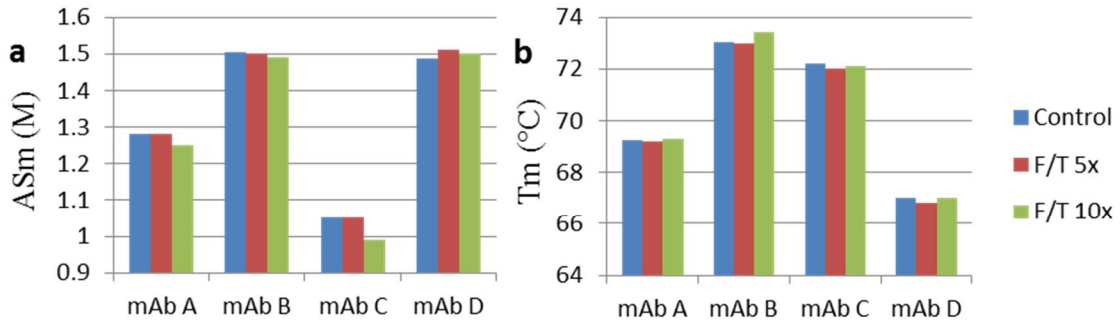

Figure 9. KBS and DSF evaluation of effects of freeze/thaw cycling. (a) No changes seen in ASm values of mAb panel. (b) No changes seen in Tm values of mAb panel.

Solution pH Exposure

The panel of mAbs was subjected to solution pH stress. The samples were

evaluated at pH 4.0, 5.5, 7.0, and 8.5 in solutions comprised of 50 mM buffering agent

and 1:10 dilution of mAb in PBS. The weakly buffered PBS pH 7.4 was verified to not

affect the pH of the control buffers at a 1:10 dilution. None of the mAbs in the panel

showed any observable precipitation at pH 4.0, 5.5, or 7.0. However mild precipitation

was observed for all the antibodies at pH 8.5. However, the precipitation was not significant enough to change the soluble protein concentration.

The pH stressed samples were evaluated by DSF and KBS and showed varying amounts of change in ASm and Tm, as a function of the pH tested (Figure 10), with calculated Tm and ASm values summarized in Table 6. Importantly, it can be seen that thermal stability and solubility vary independently. For example, Tm is reduced at low pH while ASm is increased, as compared to pH 7.4 values, for all mAbs tested. For each pH condition, the variability was compared to the control, unstressed mAbs formulated in PBS pH 7.4. There is a caveat in directly comparing the unstressed samples to other pH formulations, as the stock formulation contains 154 mM NaCl, while the pH stress formulations have a final NaCl concentration of 15 mM. The presence of NaCl as an excipient in formulation has been reported to play a role in formulation stability for some mAbs (He et al., 2010). However, based on the data presented, it appears to have minimal impact under the conditions tested, for this mAb panel.

Table 6.

ASm and Tm Values as a Function of Solution pH

Sample	pH 4.0		pH 5.5		pH 7.0		pH 7.4[*]		pH 8.5	
	ASm	Tm	ASm	Tm	ASm	Tm	ASm	Tm	ASm	Tm
mAb A	1.38	57.3	1.44	68.0	1.29	69.8	1.28	69.3	1.22	69.4
mAb B	1.54	61.0	1.67	73.9	1.54	75.0	1.50	73.0	1.50	75.8
mAb C	1.10	59.6	1.23	70.3	1.07	72.1	1.05	72.2	0.99	72.4
mAb D	1.37	50.8	1.67	63.9	1.57	65.9	1.49	67.0	1.50	66.9

Note. ASm values are reported in M units, and Tm values are reported in °C
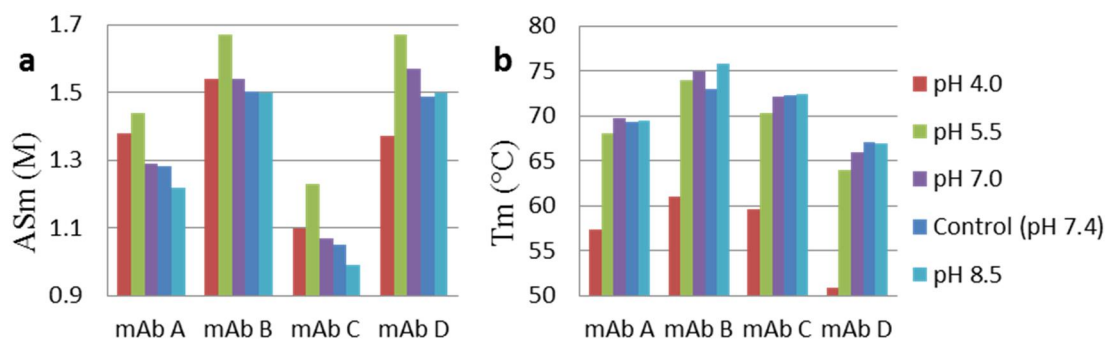* pH 7.4 values are from the unstressed mAb panel in PBS pH 7.4

Figure 10. ASm and Tm values as a function of solution pH. The panel was subjected to various pH buffered solutions at a 1:10 dilution from the stock solution, for a final concentration of 2.5 mg/mL, the pH of the buffered solution was verified to remain unchanged with the addition of PBS. The control sample is graphed into its appropriate space based on pH, rather than separated on the left, as unstressed. (a) MAbs A, B, and C exibit increased solubility in acidic conditions, mAb D has reduction in ASm at pH 4.0, slight reduction in solubility for mAbs A and C at pH 8.5. (b) Dramatic reduction in Tm for all mAbs at pH 4.0, conformation al stability appears to improve as pH increases, plateauing neutral to basic conditions.

The pH 4.0 buffered mAbs all showed a substantial reduction in Tm, with mAbs A, B, and C having a 12.0 – 12.6 °C reduction, and mAb D showing a 16 °C reduction in Tm. In addition to the change in calculated Tm, the shape of the DSF curve is altered at pH 4.0 on mAbs B and C (Figure 11), which develop a noticeable early transition shoulder, identified by the red arrows. Shoulders like this have been reported previously by He et al. (2010) as corresponding to a multi domain unfolding process, in which one domain is distinctly less thermally stable than others, and results in an early unfolding prior to that of the remaining domains. The solubility of mAb A increased slightly with an ASm increase of 0.10 M, mAbs B and C showed minimal change in ASm, and mAb D had a reduced solubility of 0.12 M (Figure 12). Overall, at pH 4.0 mAbs A, B, and C showed an increase in colloidal stability and decrease in conformational stability, while

mAb D showed a decrease in both conformational and colloidal stability, as compared to the panel at pH 7.4.

The pH 5.5 buffered mAbs also showed variance in ASm and Tm. The Tm of mAbs A, C, and D had a reduction of 1.3 – 3.1 °C, while mAb B showed a slight increase of 0.8 °C, as compared to the control at pH 7.4. Again, mAb B displayed an early transition shoulder in the DSF melt curve (Figure 11), while the other mAbs maintained a smooth thermal stability profile. At pH 5.5, the greatest shift in ASm was observed, with all mAbs having an increased solubility of 0.16 – 0.18 M. Overall, at pH 5.5 the panel of mAbs showed an increase in colloidal stability, while mAbs A, B, and D showed a reduction, and mAb C showed an increase, in conformational stability, as compared to the panel of pH 7.4.

The pH 7.0 buffered samples were similar to the pH 7.4 control material, with some minor variations. The thermal stability was slightly higher for mAb B, with an increase of 1.9 °C, while mAb A showed a minor increase in Tm of 0.5 °C. The Tm of mAb C was minimally different from pH 7.4, while mAb D had a 1.1 °C reduction in Tm. The solubility at pH 7.0 was very comparable to pH 7.4, with a slight increase in ASm of 0.08 M for mAb D. As the difference in pH of 7.0 and the control of pH 7.4 is minimal, it was not surprising that variations in ASm and Tm were also minimal. These observed variations may have been a reflection of the differences in NaCl concentration in the solution and needs to be evaluated in future studies.

The pH 8.5 buffered samples were similar to the results from pH 7.0, as the ASm value is largely unchanged, and minor reductions of 0.06 M were seen in mAbs A and C.

The thermal stability at pH 8.5 is unchanged in mAbs A, C, and D, while mAb B has an increase of 2.7 °C. Interestingly, the pH 8.5 buffered samples all showed some mild precipitation, which was not seen in any of the other pH stressed samples. The theoretical pI for the mAb panel ranges from 8.0 – 9.0. Thus the pH 8.5 buffer point likely either crossed the isoelectric point for each mAb on the panel, or came very near, thus neutralizing surface charge, triggering colloidal instability, which led to aggregation and precipitation. Buffering agent is not thought to be the cause of precipitation at pH 8.5, as reported by Yamniuk et al. (2014). Buffering agent did not affect ASm values; rather, pH was the primary variable.

Results Summary

The initial generation of the mAb panel was successful, as four of five mAbs were able to express at an acceptable rate, and could be formulated at the high concentration necessary for the study. The initial characterization performed on the panel revealed each mAb to have a unique ASm/Tm profile. A series of four stresses were then applied to the mAb panel, and insoluble aggregate formation was monitored, as well as ASm and Tm values. The ASm and Tm values were not observed to change in a definite trending manner for the transient stresses: elevated isothermal incubation, agitation induced stress, and freeze/thaw cycling. However, ASm and Tm values were observed to vary independently as a function of the solution pH.

Chapter IV

Discussion

Monoclonal antibodies are the fastest growing area in drug development. The estimated cost of discovering, developing, producing, and testing a drug quality mAb is in the hundreds of millions of dollars (Morgan, Grootendorst, Lexchin, Cunningham, & Greyson, 2001). As such, a considerable amount of attention has been directed at methods to predict biophysical characteristics that are most likely to identify a developable drug. To measure aggregation propensity, a variety of assays have been developed, each designed to monitor or predict aspects of stability, including high throughput assays like KBS and DSF. These assays monitor aspects aligned with the two key aggregation pathways, native and non-native aggregation. However, in the case of both assays, only a single metric can be attained, melting temperature (Tm) from DSF, and solubility (ASm) from KBS. Putting these values into perspective for stability as a whole is vital to comparing a panel of mAbs, and qualification for development.

A common situation in drug discovery and development is the evaluation of a panel of mAbs, each possessing the appropriate, and comparable, activity in an in vitro or in vivo model. Selecting the drug development candidate then falls to stability characterization. If this hypothetical mAb panel were to have a stability profile as seen in Figure 13a, where the mAbs rank order the same in their ASm and Tm profile, the selection of mAb B becomes obvious as it has the highest paired stability indicators.

However, if instead the panel only consisted of mAbs A, C, and D (Figure 13b), then identifying the most stable is unclear. The profile of mAb A shows a medium ASm and Tm profile, relative to the range of values from the panel. Meanwhile mAb C has a high Tm, but low ASm, and mAb D is opposite, with a low Tm, but high ASm. The ability to weigh and combine those two values into a single quantifiable metric, accounting for both aggregation pathways is needed. It is believed that a metric incorporating the propensity for the dual aggregation pathways, native and non-native, could be generated by studying a panel of mAbs and their relative sensitivity to pathway-specific stresses, as measured by DSF and KBS assays. This would then allow for the ranking of a panel of mAbs by stability.
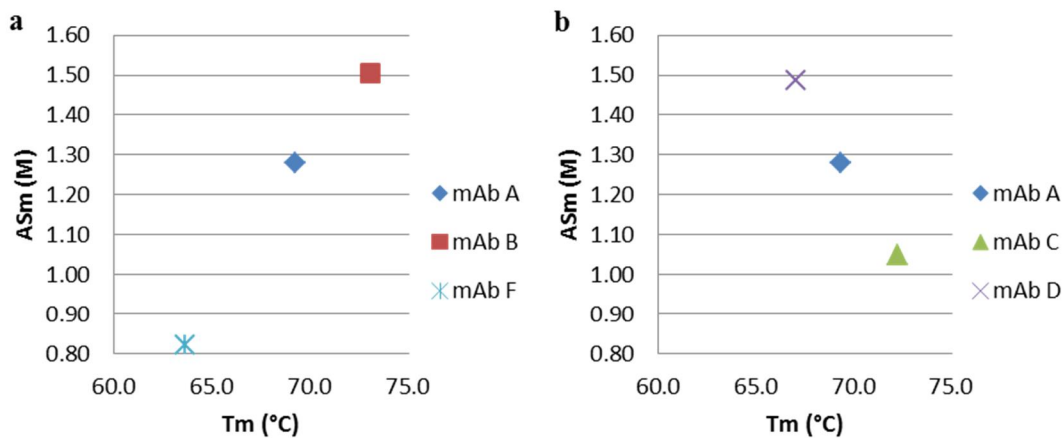

Figure 13. Comparison of ASm and Tm profiles for stability ranking. (a) Set of 3 mAbs that can easily be rank ordered for stability as ASm and Tm values rank consistently for each mAb. (b) Set of 3 mAbs that cannot be easily rank ordered for stability as ASm and Tm values do not rank order the same.

Considerable effort has gone into correlating the high throughput DSF assay with the well-established DSC standard for evaluation of protein thermal stability (He et al., 2010). Thermal stability in turn is widely accepted as a predictor of non-native aggregation propensity (Goldberg et al., 2011). Additionally, the KBS assay has been identified as a method for predicting native aggregation propensity, in a manner that exceeds the abilities of prior assays, such as ultrafiltration and dynamic light scattering (Yamniuk et al., 2013). Furthermore, assays designed to study both aggregation pathways have been used in parallel, as a tool for formulation development of mAbs (Banks et al., 2012; Goldberg et al., 2011). Also, due to the handling activities associated with the production, purification, processing, filling, shipping, and storage of mAb-based drugs, many exogenous stresses have been studied in an effort to better understand how they affect mAb aggregation. However, no reports in the current literature comment on combining these two sets of activities, artificial stress and aggregation propensity-predicting assays, in a manner designed to determine if mAbs have differential stress responses as seen by a shift in thermal stability or solubility.

To evaluate the differential stress response of mAbs the panel was exposed to a series of actions that mimicked actual stresses a drug would be subjected to during manufacturing and storage. After stress exposure, the mAbs' ASm and Tm values were obtained and compared to pre-stress values. Each stress applied involved measuring multiple time points, to monitor trending of response to stress over time, with the exception of pH, which used multiple different pH formulation levels. The aggregation pathway sensitization caused by applied stress, as observed by shifts in ASm and Tm, did not occur as expected. The stresses were successful in triggering the mAbs to enter into

the aggregation pathway, as witnessed by precipitation in samples during the stress application, but no definite shift in ASm or Tm was recorded, with the exception of the pH buffer study.

While the original stresses were designed to be pathway-specific, the fact that the stresses could be divided into two other categories went unnoticed. The stresses included elevated isothermal incubation and agitation, which are directed at non-native aggregation, and freeze/thaw cycling, and solution pH, which are reported to affect native aggregation. However, an applicable alternate categorization is transient stress and perpetual stress, as elevated isothermal incubation, agitation, and freeze/thaw cycling are all stresses that are applied, and then ceased after a predetermined amount of the stress was applied. While solution pH has a perpetual effect, as the mAb is exposed, and then left in that environment for the entirety of the evaluation.

Transient stresses cause the entry of mAbs into the aggregation pathways; this was verified by the presence of insoluble, visible precipitate in the solution, for each of the transient stresses applied to the mAb panel (Tables 3 – 5). However, it appears that as the affected proteins entered into the aggregation cascade (Figure 1), regardless of pathway, they proceeded through the nucleation event, forming agglomerated aggregates, and macro particulates. As such, the majority of molecules that were undamaged, as seen by the relatively low change in soluble protein concentration, remained in either a native undamaged state, or at least a state that was not more or less sensitive to the DSF and KBS assays (Figures 8, 9; Table 3). This is most apparent in the extreme stressing of mAbs A and D, in which incubation at 60 °C resulted in the near complete precipitation and loss of mAb D, and the loss of over half the soluble protein from mAb A. Even after

this extreme stress, the ASm and Tm values were largely unaffected. Evidence that structural damage did exist can be seen in the baseline DSF value for mAb A (Figure 7), in which the extreme heat stressed mAb showed a 5-fold increase in baseline fluorescence. This increase in baseline fluorescence suggests that the ambient hydrophobicity of the environment was higher than the unstressed control, likely caused by partial unfolding of mAb A, thus exposing hydrophobic core residues. Yet the Tm remained unaffected, suggesting that even in a partially damaged state, the mAb was not more susceptible to thermal denaturation. Hence, there was minimal change in Tm. This stress also resulted in no shift in ASm, which further confirms that colloidal stability is not directly affected by conformational stability.

Perpetual stress, or perpetual exposure, is known to affect stability, as is apparent in the substantial efforts to tailor formulation to individual mAbs (Banks et al., 2012; He et al., 2010). This was verified by the solution pH study that was performed, in which changes in ASm and Tm were seen across the entire mAb panel (Table 6). This experiment also verified that thermal stability and solubility can vary independent of each other, as can be seen in the opposite trending of ASm and Tm values, as a function of pH (Figure 14).

From the solution pH study, the comparison to the unstressed material was conceptually different than the other stresses, in two notable manners. The first has already been discussed, as the solution pH is a persistent stress, as opposed to the transient stresses applied to the mAbs. The second difference is that determining the variance from unstressed mAbs does not hold the same meaning as transiently applied stresses, as solution pH is a variable in all assays, simply unchanged, except in the

solution pH study. Because the mAb panel was in a formulation at pH 7.4, comparison to the control was arbitrary, unless being used for a formulation study, which this was not. As such, evaluating the mAb panel and their respective ASm and Tm values as a function of pH is a more logical method of analysis. As seen in Figure 14a, the thermal stability trend of the mAb panel is largely uniform, with decreased Tm values at low pH (4.0), and increasing stability, until plateauing from neutral to basic conditions.

The KBS assay showed a different trend as can be seen in Figure 14b. The mAb panel showed increased solubility in acidic conditions, with a clear peak in solubility at pH 5.5, and a steady decline in colloidal stability as the solution became neutral and shifts into a basic environment. The effect of pH on protein stability is significant, and has been studied in depth as part of formulation analysis (He et al., 2010). That the colloidal stability reduced as the pH increased above pH 5.5 was also expected, as the theoretical pI of the panel of mAbs ranges from pH 8.0 – 9.0. Thus, as the solution approaches the pI of the mAb, surface charge generated repulsions are neutralized and native aggregation, driven by hydrophobic associations, is expected. This was also confirmed by the precipitation seen in the pH 8.5 stressed samples, but not at any of the other pH levels tested. Surprising was that in the pH 8.5 environment that triggered precipitation there were only minor reductions in ASm for mAbs A and C, while mAbs B and D showed no shift, as compared to pH 7.4. Because the soluble protein concentration change was minimal, it is believed that some small percentage of each mAb had already entered into the aggregation pathway. The resulting neutralization of surface charge caused by being at or near the isoelectric point of the protein allowed for nucleation and ultimately the formation of the insoluble aggregate seen.

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Overall, the solution pH study was able to show a change in ASm and Tm values, but those changes are fairly consistent across the mAb panel, with some exceptions. For both the trending of the Tm and ASm values as a function of pH (Figure 14), the mAbs all remain in rank order, and the Tm-pH and ASm-pH curves have very similar shapes. MAb D appears to have a greater sensitivity to pH 4.0, both colloidally and conformationally, than the other members of the panel. Also, mAbs A and C share a slight downward ASm trend as pH shifts from neutral to basic, as opposed to mAbs B and D, which are level over that range. Unfortunately, this information does not provide insight into the overall stability profile of the mAbs. Rather, it showcases the benefit of these assays in formulation development.

The original goal of this study was that based on differential sensitivity to stresses, as monitored by KBS and DSF assays, a mathematical fit of the data would allow for the generation of an encompassing aggregation propensity index (APi) value. This APi value would be a metric for the incorporation of both aggregation pathways. By combining ASm and Tm values, properly weighted based on the differential stress sensitivity, APi would allow for direct comparison of stability between a collection of mAbs. The data generated at present does not lend itself to defining a logical function to generate APi, as the transient stress data and unstressed data cluster, with only two noticeable points of variation, pH 4.0 and 5.5, for each mAb in the panel (Figure 15).

While a unified metric, such as the theoretical APi value, was not able to be derived from the differential sensitivity to stress, ranking of the overall stability could be established. With the intent to rank mAbs A, C, and D based on stability (Figure 13b), observational data collected during the stresses can be used. Excluding the pH buffer

study, in which all mAbs precipitated equally, there was variance seen between stress and time to precipitation, as well as rate of precipitation. During elevated isothermal incubation mAb C showed signs of precipitation at week 1, while mAbs A and D did not (Table 3). This suggests that mAb C was more sensitive to that stress, while mAbs A and D are equal. Observations during the agitation induced stress application further confirm this trend, as again mAb C showed signs of precipitation earlier, and at a greater rate, than either mAbs A or D, which again were comparable (Table 4). Finally, during freeze/thaw cycling, mAb A began to precipitate after 2 thaws, mAb D after 3 thaws, and mAb C after 4 thaws (Table 5). While the rank order of precipitation changes during freeze/thaw, the overall sensitivity to stress can still be ranked using the three different transient stresses. Based on these data sets, mAb D appears to be the most sensitive to 2 of the 3 stresses, while mAb A is the most sensitive to one of the stresses. Using this information, a ranking of stability can be proposed: mAb C has the lowest stability and mAb D has the highest stability. The resolution of this analysis could be increased in the future by analyzing stressed material by HPLC-SEC for change in soluble aggregate and integration of particle counters to allow for a quantitative assessment of the presence of insoluble aggregate.

Efforts are being made to define what stability characteristics a mAb should have to be successfully developed into a drug. New techniques are being developed to increase the resolution and throughput of mAb aggregation propensity, such as affinity-capture self-interaction nanoparticle spectroscopy (AC-SINS) reported in the work by Estep et al. (2015). The AC-SINS assay is suggested to improve the resolution of native aggregation propensity, in a manner similar to the KBS assay. However, even with these

new assays, an arbitrary line is drawn to define a mAb's developability as acceptable or not, and scientists accept that these lines may not truly delineate a stable, developable mAb from a failure (Estep et al., 2015). These lines are drawn because mAb stability or aggregation propensity is a gradient and the best chance of success is expected to be defined by the most stable.

Unfortunately, because mAbs can be so variable, it is difficult to even establish an acceptable range of stability. The mAb panel studied had a Tm range of $67 - 73$ °C, and an ASm range of $1.0 - 1.5$ M, but there is no current context to put these values in, as they could all represent stable, developable mAbs, or not. Estep et al. (2015) studied a panel of 32 clinical stage mAbs by hydrophobic interaction chromatography and AC-SINS, and categorized 12 as having an unacceptably high self-association profile or native aggregation propensity. However, the panel studied was of clinical stage mAbs. Thus they all empirically had an acceptable stability profile, as they were developed into a drug product, a fact the authors acknowledged.

The limitations of mAb stability analysis are that companies developing drugs are largely private about internal data, for good reason. However, with the growing number of mAbs that have been approved, or are in clinical stage testing, a case study could be done that would benefit the industry as a whole. By taking mAbs that have been empirically verified as developable, by being developed, and executing a series of stability predicting assays, such as DSF and KBS, arbitrary lines can be drawn with greater confidence that statistically define a successful stability profile. The success of a study of this nature would not necessarily define a stability profile that cannot be developed; rather, the developability line would define likely success, such that an

experimental mAb with a defined stability profile above this set line is likely to be developable. A study of this scope could help save considerable amounts of time and money by mitigating the risk of attempting to develop a mAb with an undevelopable profile.

That a single metric could be generated combining propensity to aggregate along both pathways, by using differential stress as a way to add some proportional weight to each pathway, was not supported by the data because differential stress was not observed, as a function of change in ASm and Tm. The selected stresses did generate differential damage to the proteins in an observable manner, as witnessed by precipitation, which allowed for a qualitative ranking of stability. The definition of a single value metric for aggregation propensity, like APi, would be useful in comparing mAbs, but further efforts to generate this function, in a logical manner, are necessary.
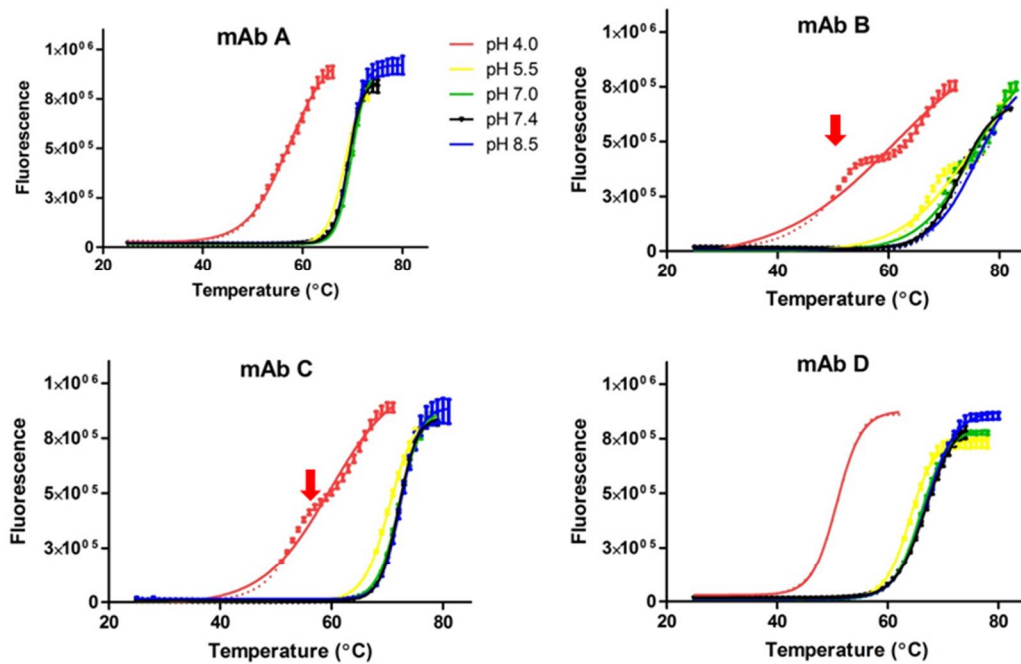
Additional Figures

Figure 11. Change in Tm as a function of solution pH. At pH 4.0 both mAbs B and C show an early stage unfolding event (red arrow), as seen by the front shoulder, mAb B also has a minor shoulder at pH 5.5. All colors correspond to the same pH, as listed in the above legend, error bars represent standard deviation.
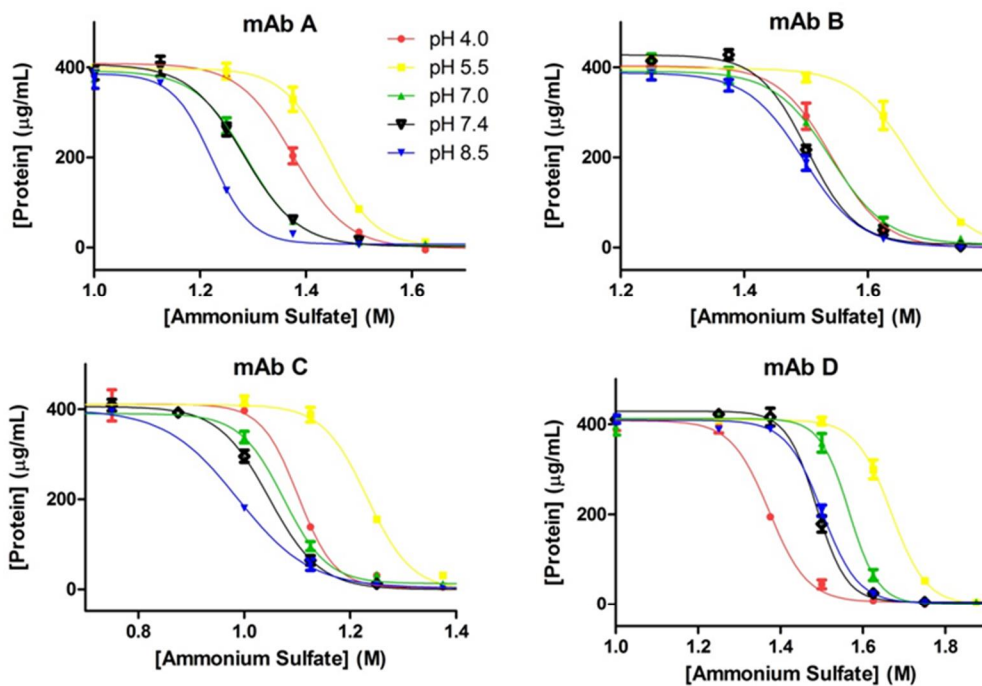
Figure 12. Change in solubility as a function of pH. KBS assay depicts the variation in solubility as seen by the gradient shifting of the sigmoidal curves. The x-axis has been adjusted to focus on the change in the partial solubility portion of the sigmoidal curve, all colors correspond to the same pH, as listed in the above legend, error bars represent standard deviation.
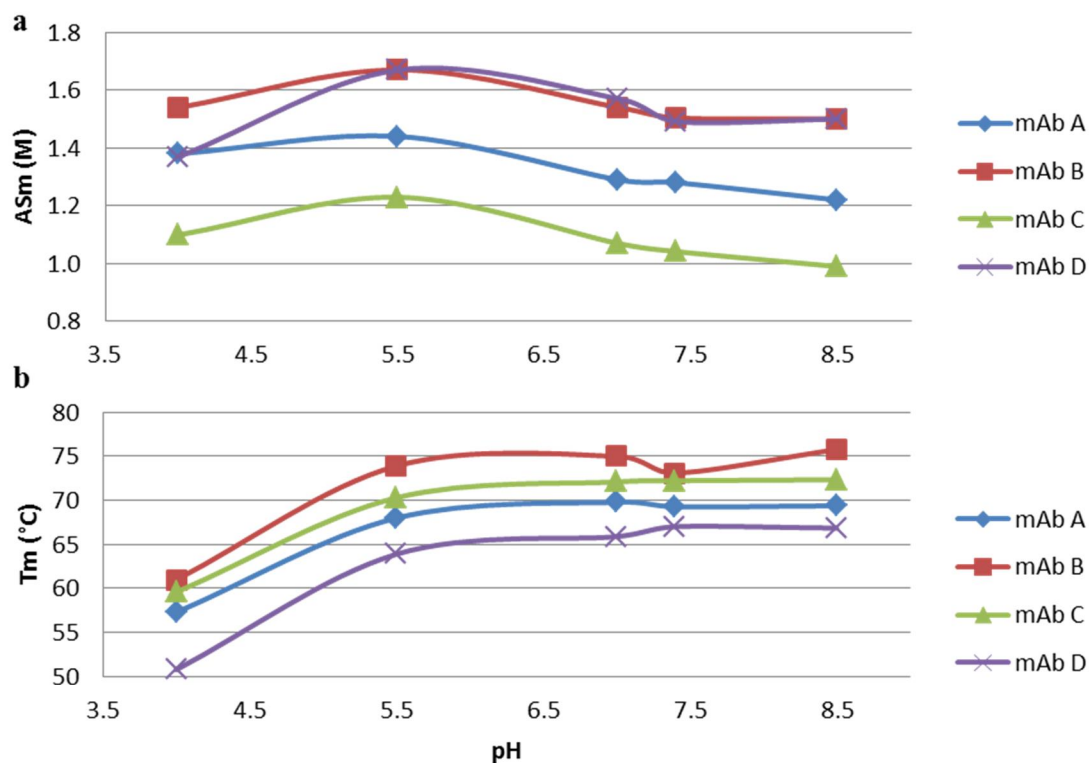
Figure 14. Trending of ASm and Tm values as a function of pH. The values listed represent the average of the calculated Tm and ASm. Rank order of both ASm and Tm remain consistent regardless of solution pH. (a) With the exception of mAb D at pH 4.0, the shift in ASm between mAbs over the pH range is uniform. (b) With the exception of mAb B at pH 7.4, which dips slightly, the Tm values steadily rise with the pH, affecting a stable Tm as the pH transitions to neutral and basic.
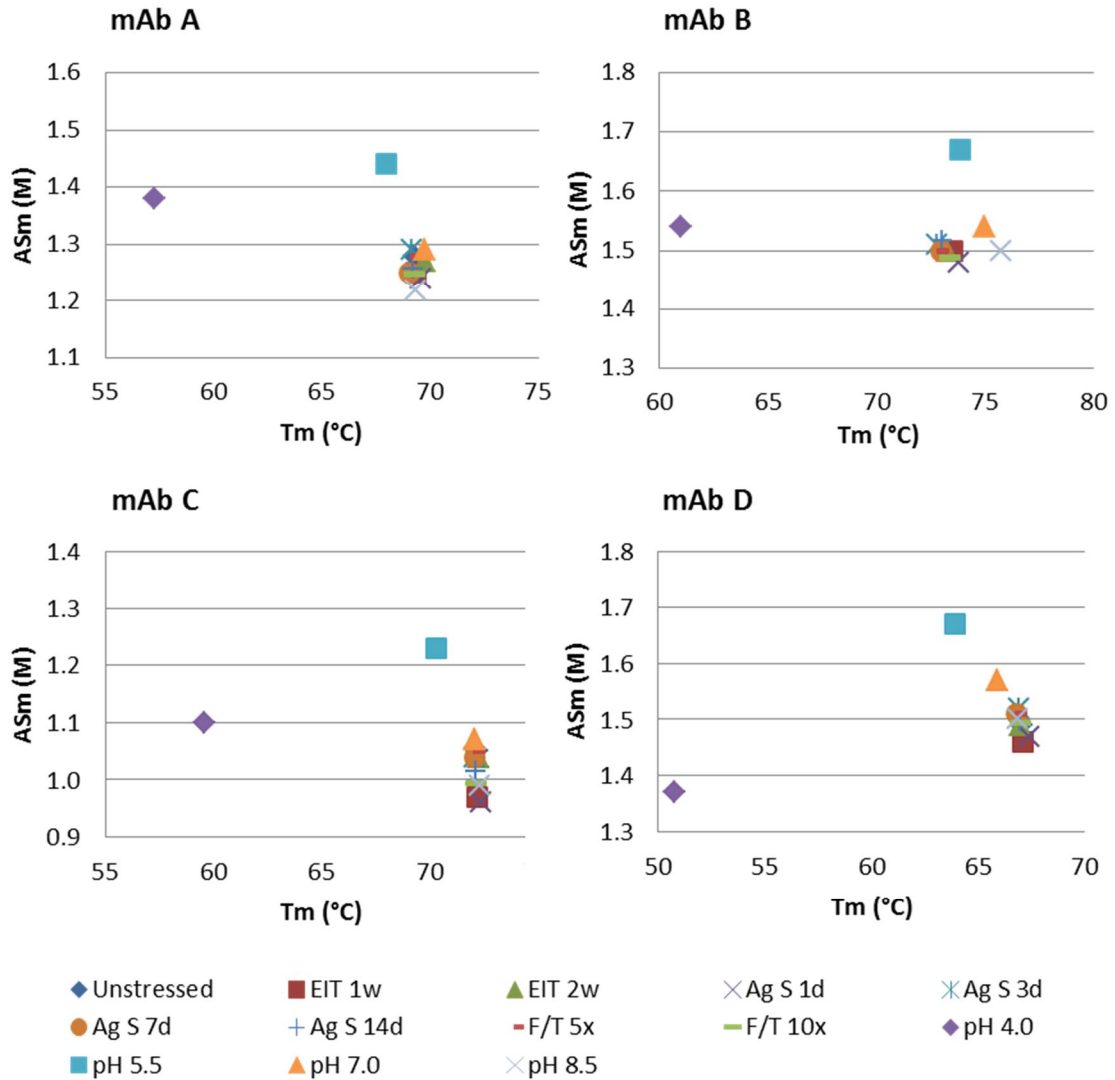
Figure 15. Summary of ASm and Tm values for all stresses applied to the mAb panel. The points displayed represent the average ASm and Tm values obtained from KBS and DSF assays. Each graph has varying x and y-axis regions, set to center the available data, but all have the same range, with a 0.5 M y-axis range, and 20 C x-axis range, thus all graphs are linearly comparable. EIT – Elevated Isothermal stress; Ag S – Agitation Stress; F/T – Freeze/Thaw Cycling; pH – Solution pH. A clustering of ASm and Tm values is seen for all of the transient stresses applied to the panel, minor shifting can be seen at neutral and basic pH values, while acidic pH exposure produced the only major shift in ASm and Tm values.

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