

Proposal for a Thesis in the Field of Biotechnology  
in Partial Fulfillment of Requirements for  
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I.

Tentative Title

“Defining a Metric for Dual Pathway Aggregation Propensity in Monoclonal Antibodies”

## II.

### Research Problem

Protein based therapeutics, especially monoclonal antibodies (mAbs), represent a fast growing sector in the therapeutic marketplace, and are projected to maintain popularity in the future (Zhang, Singh, Shirts, Kumar, & Fernandez , 2012). However, the progression of mAbs from discovery to market is time consuming and expensive, as well as rife with potential points of failure. Identifying mAbs that are especially resistant to degradation during manufacturing, transportation, storage, and administration is essential for a viable protein therapeutic.

Aggregation is widely considered one of the key concerns with respect to therapeutic mAbs, and it is vital to identify unstable constructs early in the development phase to reduce potential failure (Yamniuk et al., 2013). Aggregation of mAbs can be responsible for jeopardizing product integrity, as there is an increasing belief that aggregated proteins can increase the risk of triggering a host immune response, targeting both the aggregate as well as the active monomer (Roberts, 2014). This can reduce the efficacy of the drug, shorten the in vivo half-life, and can be potentially harmful to the patient (Lauer et al., 2012).

Aggregation occurs along two pathways, native and non-native, which can work either independently or collaboratively. The non-native aggregation pathway is initiated by conformational instability, or partial unfolding of the monomer, leading to exposure of core hydrophobic residues. This in turn interacts with other partial folded proteins, forming irreversible aggregates. The native aggregation pathway is caused by properly folded monomers associating with other monomers in an initially reversible aggregation process. However, through this close proximity and a combination of surface charge, surface hydrophobicity, and propensity to form  $\beta$ -sheets, this association ultimately becomes irreversible (Lauer et al., 2012).

Aggregation composes one of the key challenges in identifying a lead candidate for drug development, which is finding a construct with an acceptable stability profile (Yamniuk et al., 2013). To some degree, there exists a tendency toward both aggregation pathways in all proteins (Lu et al., 2012). While formulation and mutagenesis can be effective in mitigating some of the propensity toward aggregation, not all mAbs can be successfully stabilized for use as a drug (Bhambhani et al., 2012; Lauer et al., 2012). Thus, it is vital to the success of a project to be able to identify potential aggregation issues and understand their aggregation pathways.

It has been proposed that the study of both aggregation pathways in parallel would be highly useful in comparing mAb drug candidates (Yamniuk et al., 2013). However, due to the potentially large number of mAb constructs, and limited resources, the use of high throughput, low protein consumption assays are highly useful in the proper characterization of aggregation propensity. This project strives to evaluate, compare, and contrast the aggregation propensity of a panel of mAbs as studied by two high throughput assays, differential scanning fluorimetry (DSF) and kosmotrope-based solubility (KBS). DSF measures thermal stability, which is indicative of non-native aggregation propensity, and KBS reports propensity for protein association and aggregation in the native state. The goal of this study will be to evaluate these assays and their ability in gauging and assessing protein stability.

Current literature tends to focus on a single aggregation pathway, at great detail, while generally ignoring the alternate pathway (He, Hogan, Latypov, Narhi, & Razinkov, 2010; Shi, Semple, Cheung, & Shameem, 2013; Yamniuk et al., 2013), or in other instances focusing on both pathways as a means for excipient selection in formulation design (Banks et al., 2012; Goldberg, Bishop, Shah, & Sathish, 2011). My proposed study is to focus on both aggregation pathways, using the previously mentioned assays, applied to a panel of four mAbs. This panel of

mAbs will be generated and biophysically and biochemically characterized, then used as test cases for the study of aggregation propensity. To gain a full understanding of a mAb's propensity for aggregation, along either pathway, stresses believed to exacerbate each pathway specifically will be applied, with the results compared to the unstressed material. To evaluate propensity of non-native aggregation, the panel of mAbs will be subjected to elevated heat incubation, and agitation, both reported to affect protein conformational stability (Kiese, Pappenberger, Friess, & Mahler, 2008; Mahler, Friess, Grauschopf, & Kiese, 2009). While evaluation of native aggregation propensity will be conducted through pH, and repeat freeze/thaw cycle induced stress, both are believed to increase native state protein-protein interactions (Hawe, Kasper, Friess, & Jiskoot, 2009; Yamniuk et al., 2013, Zhang et al., 2012). The results from both assays and all stress conditions will be compared and contrasted. My hypothesis is that both methods will provide valuable information in assessing stability along the different aggregation pathways. That a functional metric, exemplifying a mAb's propensity for aggregation, that takes both aggregation pathways into account can be derived and used in directly comparing the total aggregation propensity of a panel of mAbs.

### III.

#### Definition of Terms

“Aggregation”: the non-covalent association of two or more monomeric proteins.

“Aggregation propensity”: inclination of a protein toward formation of oligomeric structures.

“Aggregation pathway”: the process by which the oligomeric structures are formed.

“Antibody”: a tetrameric glycoprotein comprised of two heavy chains and two light chains, approximate molecular weight of 150 kDa, key regions of interest include the constant regions C<sub>H2</sub> and C<sub>H3</sub>, and the antigen binding domain (Fab).

“Anti-Drug response”: a response by the host immune system generated specifically against an administered drug, can result in loss of efficacy or reduction in potency of the drug.

“Conformation”: the folded state of a monomeric protein.

“Degradation”: the physical or chemical process of deterioration of a monomeric protein.

“Differential Scanning Fluorescence (DSF)”: monitoring change in fluorescent signal as a function of temperature, denotes protein conformational stability.

“Drug candidate”: a molecule identified during drug discovery efforts, and selected for development.

“Excipient”: a molecule added to the formulation of a protein to enhance physical or chemical stability.

“Fab”: the unique fragment of an antibody responsible for binding to a specific antigen.

“Formulation”: solution that a drug is stored in, generally pH controlled by buffering agents and containing excipients designed at stabilizing the molecule.

“Hydrophobic core”: a series of amino acids that comprise the peptide backbone of a protein, sheltered from solvent due to their predominantly hydrophobic side chains.

“Kosmotrope based solubility (KBS)”: kosmotropes are solution based, protein structural stabilizers. Highly charged ionic kosmotropes, such as Ammonium Sulfate, are soluble at high concentrations, and can occlude proteins from solution by forcing native state association.

“Melting point”: the point at which a protein is halfway between native conformation and complete denaturation as a factor of thermal stress.

“Monoclonal antibodies”: identical antibodies generated from a single clone.

“Native aggregation”: aggregation that initiates with reversible association of two or more monomers in their native conformation.

“Non-Native aggregation”: aggregation that initiates with the partial unfolding of a protein, generally irreversible.

“Physicochemical profile”: how the physical and chemical properties of a protein affect its stability.

“Precipitate”: the phase separation of an agglomerated aggregate from soluble to insoluble.

“Stability”: the ability of a protein to maintain its native conformation.

“Stress”: a physical or chemical force exerted on a protein that can cause degradation.

## IV.

### Background of the Problem

#### 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 that mAbs are increasing in popularity is their great versatility, with FDA-approved (or approval pending) mAbs targeting a variety of diseases including multiple types of cancer, rheumatoid arthritis, multiple sclerosis, Alzheimer's disease, and other orphan diseases with no current mode of treatment (Reichert, 2013). One of the reasons that mAbs are effective against such a diverse set of diseases is the flexibility of their mechanism of action, which includes incapacitation of the target, host complimented cytotoxicity, and antibody-drug conjugates (Leader, Baca, & Golan, 2008). In addition to the broad range of diseases mAbs can treat, there is a broad range of methods in which mAbs can be developed, including hybridoma development, phage display, and in silico based rational design (Lu et al., 2012). The technology behind therapeutic mAb development is complex, but ultimately worthwhile as this next generation of drugs is created.

The first monoclonal antibody drug for immune modulation following transplants was approved by the FDA nearly 30 years ago, but the areas in which mAbs are used has increased dramatically. 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 trials, 19 of which were not for treatment of cancer (Reichert, 2013). This showcases the

flexible use of mAbs from a therapeutic standpoint, but it is the wide-ranging mechanism of action that allows for such highly diverse use in medicine.

All therapeutic mAbs have a specific target, or in the case of some mAbs currently in clinical testing, up to four targets, which the mAb binds through its variable region (Reichert, 2013). For mAbs that are effector function-mediated, interaction with immune cells through the constant region (Fc) is imperative. Therapeutic mAbs act through several methods; the mAb can bind to a desired 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 sophistication of mAbs increases, so does the methods used to find and refine these potential drug candidates.

The immune system is responsible for the production of antibodies on an as needed basis. In a normal human, there is an estimated  $10^{10}$  differently targeted mAbs (Fanning, Connor, & Wu, 1996). Thus, identification of the mAbs of interest has become an increasingly creative and refined process. Several different processes are currently in use, including hybridoma development. In this method a model organism, generally a mouse, is injected with the target protein of interest, and antibodies against that target are created, and evaluated *ex vivo* for the desired characteristics. Orthogonally, phage display allows for the high throughput screening of a library of mAbs for binding to a specific target, allowing for high resolution protein sequencing of the mAbs. Finally, one of the newest methods for mAb development is the *in silico* docking process, or rational design. Computer algorithms attempt to predict the folded structure of

proteins based on their primary sequence and then design the variable region of a mAb for complimentary shape, thus allowing binding (Lu et al., 2012).

The technology behind mAb development is improving rapidly. As this process becomes faster and more effective, the variety of uses for mAbs will increase. Because of the versatility of mAbs both in their near infinite binding capability and their multiple mechanisms of action, the use of mAbs as drugs is increasing. Therapeutic mAbs are becoming more refined, as is the technology used to select and develop them as drug candidates. With this increase in the use of mAbs as drugs comes the need for improved characterization, and the ability to select the best candidates for development and progression into clinical testing.

#### Stability Concerns with Monoclonal Antibodies

As with any drug, purity and stability is vitally important, and protein based drugs, including mAbs, are no different. 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, 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).

Evidence has arisen that the presence of aggregated proteins can 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, which is highly problematic as several mAb based drugs are used for chronic diseases. More seriously is the potential, albeit very rare, possibility that aggregated

material can trigger an autoimmune event, such as red cell aplasia, in which a patient's system begins targeting endogenous proteins (Roberts, 2014). Because stability is so important, and aggregation can be so problematic, 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).

### Protein degradation pathways

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 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.

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 that it becomes insoluble and forms visible particulate, or precipitation (Manning et al., 2010; Roberts, 2007). 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. Significant efforts are

made to minimize a protein's propensity to enter into either of the aggregation pathways (Roberts, 2014). Thus, by appropriately screening mAbs early in the development process, and focusing on both aggregation pathways, the efforts necessary to minimize aggregation through alternate means, such as formulation and mutagenesis can be reduced dramatically.

### Native State Aggregation

Native state aggregation is the aggregation 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  $\beta$ -sheets and  $\alpha$ -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  $\beta$ -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 that conformation is a  $\beta$ -sheet, forming 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 State Aggregation

Non-native aggregation is the aggregation 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.

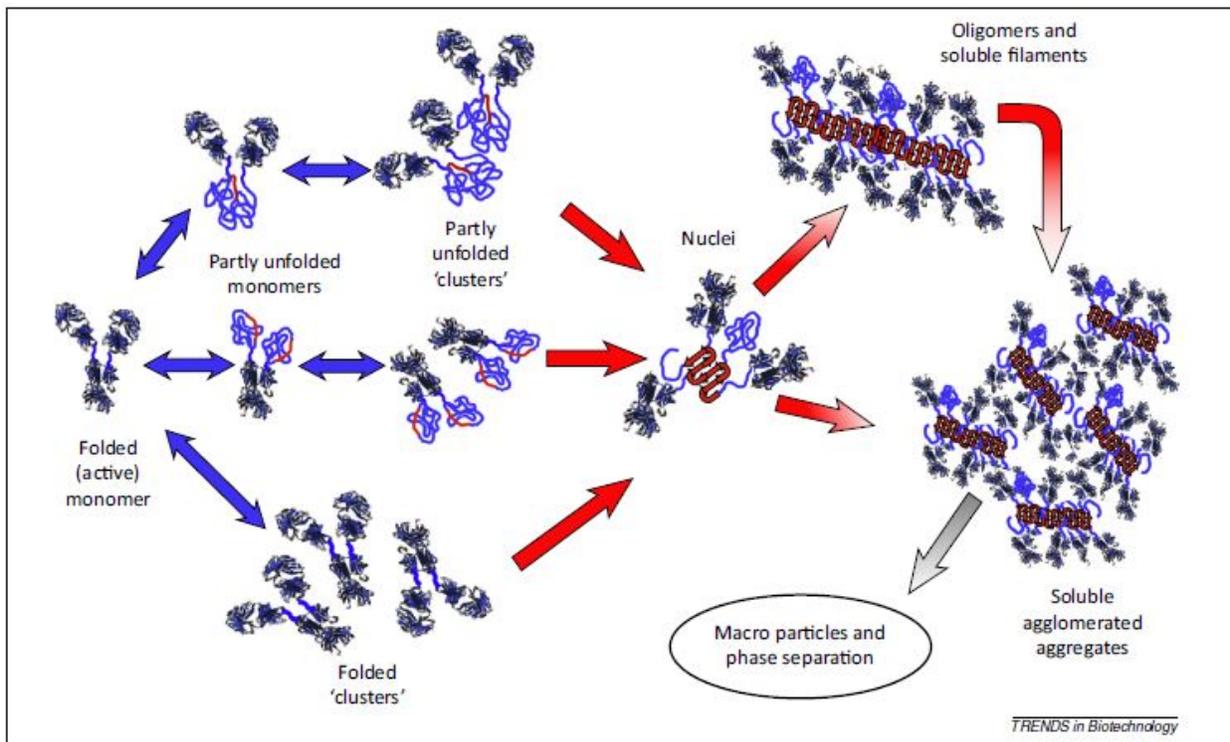


Figure 1. Native and non-native aggregation pathways, including progression to nucleation, soluble filaments and/or agglomerates, and precipitation. From “Therapeutic protein aggregation: mechanisms, design, and control” by C. J. Roberts, 2014, *Trends in Biotechnology*, 32(7), p. 373. Copyright 2014 by Elsevier Ltd.

## Formulation and Mutagenesis

Inherent stability is highly desirable for any drug candidate. However, as previously mentioned, it is widely accepted that proteins are not exceptionally stable in their native conformation. 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 neutralizes them through mutation of hopefully innocuous residues. Both methods can be used to improve protein stability. However, neither can completely prevent protein degradation.

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 be able to 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). The drawback to a mutagenesis based approach is that if done too late in the development cycle, many data sets may need to be repeated with the mutated protein to ensure drug efficacy is maintained. Additionally, most mAbs have reasonably high sequence similarity, with the exception of the variable region, which is unique to each mAb and what engenders its binding profile. The variable region of a mAb tends to have the highest risk of inducing aggregation. Thus, mutagenesis studies have been conducted and have shown some success at reducing aggregation without affecting efficacy, but obviously this is not always the case (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.

## Current Approaches to Aggregation Monitoring

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 (DSL). 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, DSL 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 DSL 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, and 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.

## Thermal Stability is Indicative of Non-Native 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 ( $T_m$ ) or hydrophobic exposure temperature ( $T_h$ ) (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,  $\mu\text{g}$  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.

### Kosmotrope Based Solubility is Indicative of Native State Aggregation Propensity

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  $\beta$ -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, 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 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).

#### Joint Study of Native and Non-Native Aggregation as a Method to Define Total Aggregation Propensity

Significant work has been invested in studying protein aggregation, although in many situations those efforts tend to focus on a single aggregation pathway, such as 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 focused 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 the ability to clinically develop a mAb, from a stability standpoint. Ultimately, there has been little focus on characterizing both aggregation pathways. Which begs the question, is that important? My hypothesis is that through careful evaluation of the propensity of a mAb for both aggregation pathways, a metric joining those

propensities can be derived, leading 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  $T_m$ , 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  $AS_m$ , 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, by which I mean, having a 3 °C higher melting point as measured by DFS is difficult to classify on its own, or even if compared side-by-side with KBS result that shows a 0.2 M difference in  $AS_m$ . How significant is 3 °C  $T_m$ , or 0.2 M  $AS_m$ , 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 identifying instabilities in the biophysical properties of a mAb will help to identify candidates that may not have the potential for development.

However, simply determining the  $T_m$  and  $AS_m$  of a mAb will not necessarily convey the true stability. Instead, it conveys that stability in a single situation. Thus, I am proposing a study of path specific aggregation propensity under unstressed and stressed conditions. The stresses proposed will be those that are believed to exacerbate individual pathways specifically. This is not a formulation screening process but rather an empirical evaluation of multiple mAbs to define a correlative metric, taking into account both native and non-native propensities. This

metric could then be used to directly compare multiple mAbs by their propensity for aggregation in a more quantifiable manner. Formulation will always play a role in the developmental success of a mAb. However, by identifying and excluding unstable mAbs early on we reduce the potential for investing significant resources into a mAb, which cannot be stabilized through the addition of excipients (Lauer et al., 2012).

## V.

### Research Method

The goal of this study is to gain insight into mAb aggregation propensity along both aggregation pathways. To this end, a selection of 4 human mAbs with uniform C<sub>H</sub>2 and C<sub>H</sub>3 domains but distinctly different Fab domains will be selected for use as a sample panel of mAbs for aggregation propensity analysis. Variation in the Fab will be determined by sequence alignment of the CDR region of the mAbs. The panel will be expressed in mammalian cell culture, purified by Protein A affinity chromatography, and stored in 10 mM Phosphate, 150 mM NaCl pH 7.4 (PBS). Expected amount of each mAb needed for all planned studies is 25 mgs, if the necessary quantity of mAb is not available from initial transfection, then repeat transfections will be completed to attain the desired quantity of mAb. If a mAb is incapable of attaining an acceptable expression rate, then it will be replaced by an alternate construct. Each mAb will be concentrated to a stock protein concentration of  $25 \pm 1$  mg/mL. Some mAbs have difficulty handling high solution concentrations. If a mAb is incapable of attaining high concentration, then it will be replaced by an alternate construct. Baseline characterization will be performed on all mAbs. This will include HPLC-SEC for purity evaluation, DSF for initial melting point

determination, and KBS for AS solubility establishment. The mAb panel will be stored at 4 °C when not in use, for the duration of the study.

The two high throughput assays that will be used to evaluate the propensity for native or non-native aggregation will be DFS and KBS. DFS will use SYPRO® Orange dye (Life Technologies, Woburn, MA), to evaluate conformational stability. Samples will be tested in duplicate by RT-PCR as described by He et al. (2010). A blank of the dye and the formulation that the evaluated mAbs are in will be run in duplicate and subtracted from the thermal data. The melting temperature ( $T_m$ ) of each sample will be calculated by fitting the thermal profile with a Boltzman Sigmoidal curve. The Boltzmann curve fit establishes a constant maximum and minimum signal, from which the equation can fit the midpoint value, which is represented by  $T_m$ , reported in °C.

The KBS assay will be performed as described by Yamniuk et al., (2013). Briefly, AS dissolved in the buffer matching tested mAbs will be prepared, in a dilution series. The concentration of mAb will be held constant, and upon addition to the AS dilutions, the mAb will precipitate out as a function of AS solubility. MAb remaining in solution will be quantified by A280 on a NanoDrop Spectrophotometer. All samples will be tested in duplicate, and for each sample tested there will be a corresponding blank that is composed of only AS and buffer, no mAb, to be used as a blank for the NanoDrop. The data will be fit to a Boltzman Sigmoidal curve and the midpoint ( $AS_m$ ) will be used as the comparison point.

To stress the mAb panel in a manner directed at affecting non-native aggregation propensity two conditions will be examined, elevated isothermal incubation and agitation induced stress. Elevated isothermal stress will be applied by incubating the mAbs at least 15 °C

below the calculated  $T_m$ , for one week. Agitation stress will be applied by horizontally shaking the mAb solution at 200 rpm at room temperature for one week, taking samples for analysis at 24, 72, and 168 hours, as described by Kiese et al. (2008). For both stresses presence of precipitation and changes in concentration will be noted. If protein loss is too great and DFS and KBS assays cannot be completed, then those mAbs will be repeated at lower shaking speed, or shorter incubation period, three days. However, if no discernable change in DFS or KBS profile is seen, then the stress can be extended through amount of time shaken, or length of isothermal incubation.

To stress the mAb panel in a manner directed at affecting native aggregation propensity two conditions will be examined: solution pH and freeze/thaw cycling (F/T). To evaluate the effects of pH on the mAb panel, buffers will be prepared at four pH levels: 4.0, 5.5, 7.0, and 8.5. The stock mAb solutions will be spiked into the various pH solutions. Buffer samples without mAb will be prepared to determine the actual solution pH, and to be used as blanks for both assays. The freeze/thaw stress will be conducted as described by Hawe et al., (2009) for a total of 5 cycles. If protein loss is too great, or if no change in the DFS or KBS profile is seen, then the pH range will be expanded, or the number of F/T cycles will be adjusted.

	Logic	Experiment	Control and Why
1.	Agitation is believed affect the conformational stability of a mAb, which should increase its propensity for non-native aggregation, while not affecting native aggregation.	Shake mAb panel and take time point samples to evaluate by KBS and DSF assays.	Non-stressed mAbs, contrast difference between stressed and non-stressed mAbs, and apparent differences between DSF and KBS results.
2.	Thermal incubation (below $T_m$ ) has been attributed to non-native aggregation pathways, however the increased temperature will likely increase solution based interaction of native monomers, thus may affect native aggregation propensity.	Incubate mAb panel at increased temperature for 1 week and evaluate by KBS and DSF assays.	Non-stressed mAbs, contrast difference between stressed and non-stressed mAbs, and apparent differences between DSF and KBS results.
3.	Solution pH is responsible for total protein charge, thus if that charge is neutralized it should increase native aggregation, while the effects on conformational stability vary widely.	Dilute mAb panel in range of pH buffered solutions and evaluate by KBS and DSF assays.	No mAb solution treated in same manner to control actual final pH, and act as blank. Non-stressed mAbs, contrast difference between stressed and non-stressed mAbs, and apparent differences between DSF and KBS results.
4.	F/T is believed to affect native aggregation propensity as seen by native state aggregates, thus should affect conformational stability, or the non-native aggregation pathway.	Repeat F/T of mAb panel and evaluate by KBS and DSF assays.	Non-stressed mAbs, contrast difference between stressed and non-stressed mAbs, and apparent differences between DSF and KBS results.

## VI.

### Limitations

While the CDR region of mAbs only comprises approximately 10% of the total structure, the physicochemical profile of mAbs can be distinctly different (Goldberg et al., 2011; Yamniuk et al., 2013). In combination with the nearly unlimited combinations of sequences that can define a mAb, no finite panel can be exemplary of all permutations.

Using high throughput screening assays allows for greater sample processing and evaluation, but the resolution is not as high as other methods. Thermal melt temperatures calculated by DSF are comparable to DSC. However, because DSF is lower resolution the number of transition states and order, is not clear, which limits the insight that can be gained from the results. Also, focusing on the aggregation propensity pathway will not give any insight into the other physical degradation pathways, nor will it recognize propensity for chemical degradation.

There are many stresses that a mAb may be subjected to during the production: filling, packaging, and storage as a drug product, any of which can trigger aggregation along either pathway. The stresses selected for use, to model aggregation propensity, may overlook or marginalize significant aggregation sensitivity. Thus, there may be other stresses that are more indicative of sensitivity to either aggregation pathway.

The overall design of this study is to enable prediction of a mAb's stability profile, which does not necessarily correlate with success in formulation stabilization. A correlation has been drawn between higher native state stability and easier formulation stabilization, but this study does not attempt to address that issue (Yamniuk et al., 2013).

VII.

Tentative Schedule

First submission of thesis proposal .....	November 5, 2014
Draft I Proposal returned for revision.....	December 8, 2014
Draft II Proposal returned for revision.....	January 1, 2015
Final Proposal submitted.....	February 1, 2015
Thesis Director agrees to serve .....	February 15, 2015
Complete data generation .....	June 1, 2015
First draft completed.....	July 1, 2015
Thesis director returns corrected first draft.....	July 15, 2015
Revised draft completed .....	August 1, 2015
Thesis director returns revised draft .....	August 15, 2015
Final text submitted to thesis director and research advisor .....	September 1, 2015
Final text approved .....	September 15, 2015
Bound copy submitted .....	October 15, 2015
Graduation.....	November 2015

## VIII.

### References

- Alsenaidy, M. A., Jain, N. K., Kim J. H., Middaugh, C. R., & Volin, D. B. (2014). Protein comparability assessments and potential applicability of high throughput biophysical methods and data visualization tools to compare physical stability profiles. *Frontiers in Pharmacology*, 5(39), 1-19. doi: 10.3389/fphar.2014.00039
- Reviews the challenges of producing a protein therapeutic, and the necessary monitoring of stability throughout all handling processes.
  - Summarizes multiple non-traditional visualization techniques that combine parallel stability monitoring assays.
- Andrews, J. M., & Roberts, C. J. (2007). A Lumry-Eyring nucleated polymerization model of protein aggregation kinetics: 1. Aggregation with pre-equilibrated unfolding. *Journal of Physical Chemistry B*, 111, 7897-7913. doi: 10.1021/jp070212j
- Study of the stability and irreversible nature of non-native state aggregates.
  - Mathematical modeling of non-native aggregation, believed to widely applicable, incorporating both the Lumry-Eyring and nucleated polymerization models.
- Banks, D. D., Latypov, R. F., Ketchem, R. R., Woodard, J., Scavezze, J. L., Siska, C. C., & Razinkov, V. I. (2012). Native-State solubility and transfer free energy as predictive tools for selecting excipients to include in protein formulation development studies. *Journal of Pharmaceutical Sciences*, 101, 2720-2732. doi: 10.1002/jps.23219
- Study of native and non-native aggregation pathways as a means to screen for formulation development.
  - Describes multiple techniques for protein stability monitoring, such as CD, DSC, and HPLC-SEC.
- Bhambhani, A., Kissmann, J. M., Joshi, S. B., Volkin, D. B., Kashi, R. S., & Middaugh, C. R. (2012). Formulation design and high-throughput excipient selection based on structural integrity and conformational stability of dilute and highly concentrated IgG1 monoclonal antibody solutions. *Journal of Pharmaceutical Sciences*, 101, 1120-1135. doi: 10.1002/jps.23008
- Non-Native state aggregation monitoring as a predictor for successful formulation development, used pH and elevated temperature screening in evaluating a single mAb.

- Integrated empirical phase diagrams as a visualization tool for orthogonal data sets.

Cafilisch, A. (2006). Computational models for the prediction of polypeptide aggregation propensity. *Current Opinion in Chemical Biology*, 10, 437-444. doi: 10.1016/j.cbpa.2006.07.009

- Discusses the design of computational aggregation models, including the physical characteristics taken into account, and the states of conformational changes modeled.

Corrigan P. A., Cicci, T. A., Auten, J. J., & Lowe, D. K. (2014). Ado-trastuzumab emtansine: a HER2-positive targeted antibody-drug conjugate. *Annals of Pharmacotherapy*, 48, 1484-1493. doi: 10.1177/1060028014545354

- Discusses the concept and mechanism of action of an antibody-drug conjugate.

Costanzo, J. A., O'Brien, C. J., Tiller, K., Tamargo, E., Robinson, A. S., Roberts, C. J., & Fernandez, E. J. (2014). Conformational stability as a design target to control protein aggregation. *Protein Engineering, Design & Selection*, 27(5), 157-167. doi: 10.1093/protein/gzu008

- Attempted to identify the rate limiting step in the non-native aggregation process, protein unfolding from native state, or post-unfold formation of an irreversible aggregate.
- Utilized mutagenesis to stabilize both native state conformation and the partially unfolded conformation.
- Reinforced that the non-native aggregation pathway is not the only factor controlling aggregation.

Fanning, L. J., Connor, A. M., & Wu, G. E. (1996). Short analytical review: Development of the immunoglobulin repertoire. *Clinical Immunology and Immunopathology*, 79(1), 1-14. doi: 10.1006/clin.1996.0044

- Review of the natural production of IgG, and the natural role of autoantibodies in autoimmune disorders.

Fincke, A., Winter, J., Bunte, T., & Olbrich, C. (2014). Thermally induced degradation pathways of three different antibody-based drug development candidates. *European Journal of Pharmaceutical Science*, 62(1), 148-160. doi: 10.1016/j.ejps.2014.05.014

- Describes methods for elevated temperature induced degradation, accelerated stability analysis, and assays used to monitor that degradation.
- Found that mAbs form heterogeneous solutions, with multiple aggregate species, and degradation species as a factor of the environment, and mAb itself.

Gibson, T. J., McCarty, K., McFadyen, I.J., Cash, E., Dalmonte, P., Hinds, K. D., Dinerman, A. A., Alvarez, J. C., & Volkin, D. B. (2011). Application of a high-throughput screening procedure with PEG-induced precipitation to compare relative protein solubility during formulation development with IgG1 monoclonal antibodies. *Journal of Pharmaceutical Sciences*, 100, 1009-1021. doi: 10.1002/jps.22350

- Describes the mechanism of action behind PEG-induced precipitation, and the added utility for study of native aggregation. Suggests comparable function to ammonium sulfate precipitation, further entrenching it as a valid method for monitoring native aggregation propensity.

Goldberg, D. S., Bishop, S. M., Shah, A. U., & Sathish, H. A. (2011). Formulation development of therapeutic monoclonal antibodies using high-throughput fluorescence and static light scattering techniques: Role of conformational and colloidal stability. *Journal of Pharmaceutical Sciences*, 100, 1306-1315. doi: 10.1002/jps.22371

- Utilizes two high-throughput screening assays, DSF and DSLS, to study the aggregation propensity of a four mAb panel, establishing these assays as beneficial for formulation studies.
- Draws a strong correlation between DSF and DSC data, giving additional support for the use of DSF as a method to monitor conformation stability. Also suggests that these assays, under accelerated stability conditions, are predictive of real time stability.

Hawe, A., Kasper, J. C., Friess, W., & Jiskoot, W. (2009). Structural properties of monoclonal antibody aggregates induced by freeze-thawing and thermal stress. *European Journal of Pharmaceutical Sciences*, 38(2), 79-87. doi: 10.1016/j.ejps.2009.06.001

- Defined a freeze/thaw stress based assay to monitor protein stability, found appropriate number of cycles to result in protein damage, and soluble aggregate, but limited insoluble precipitate.
- Suggests that the aggregates formed from various artificial stress factors have different physicochemical properties, such as covalent vs non-covalent linkages, and particulate conformation.

He, F., Hogan, S., Latypov, R. F., Narhi, L. O., & Razinkov, V. I. (2010). High throughput thermostability screening of monoclonal antibody formulations. *Journal of Pharmaceutical Sciences*, 99, 1707-1720. doi: 10.1002/jps.21955

- Extensive formulation study and comments on the effects of excipients on mAb stability, as function of conformational stability.
- Established correlative data between the first transition temperature as measured by DSC and the hydrophobic exposure temperature measured by DSF.

Hwang, W. Y. K., & Foote, J. (2005). Immunogenicity of engineered antibodies. *Methods*, 36(1), 3-10. doi: 10.1016/j.ymeth.2005.01.001

- Discusses the potential for generating anti-drug antibodies from monomeric mAbs, and methods to reduce this immunogenic response.

International Conference on Harmonisation; Final guidelines on stability testing of biotechnological/biological products. (1996). *Federal Register*, 61, 36465-36469. Retrieved from <http://www.fda.gov/OHRMS/dockets/dockets/05d0047/05d-0047-bkg0001-Tab-07.txt>

- Defines the guidelines for protein monitoring, and makes suggestions to necessary stability profiles.

Johnson, C. M. (2013). Differential scanning calorimetry as a tool for protein folding and stability. *Archives of Biochemistry and Biophysics*, 531(1-2), 100-109. doi: 10.1016/j.abb.2012.09.008

- Covers the fundamentals of DSC as a tool for monitoring protein conformational stability.

Kiese, S., Pappenberg, A., Friess, W., & Mahler, H. C. (2008). Shaken, not stirred: Mechanical stress testing of an IgG1 antibody. *Journal of Pharmaceutical Sciences*, 97, 4347-4366. doi: 10.1002/jps.21328

- Studied the different effects of shaking and stirring on mAb stability, in the presence and absence of surfactants.
- Found significant differences on the state of the aggregates as a function of shaking, which produced soluble aggregate, and stirring which produced considerable insoluble aggregates.

Kramer R. M., Shende, V. R., Motl, N., Pace, C. N., & Scholtz, J. M. (2012). Toward a molecular understanding of protein solubility: Increased negative surface charge

correlates with increased solubility. *Biophysical Journal*, 102, 1907-1915. doi: 10.1016/j.bpj.2012.01.060

- Examined a molecular approach to understanding protein solubility by focusing on native aggregation.
- Found a correlation between negative surface charges and increased solubility. As well as a correlation between AS and PEG induced native aggregation propensity.

Lauer, T. M., Agrawal, N. J., Chennamsety, N., Egodage, K., Helk, B., & Trout, B. L. (2012). Developability index: A rapid *in silico* tool for the screening of antibody aggregation propensity. *Journal of Pharmaceutical Science*, 101(1), 102-115. doi: 10.1002/jps.22758

- Presents an *in silico* molecular modeling tool for predicting aggregation propensity, termed the developability index score.
- Identify key attributes of the protein that are included in their algorithms for predicting aggregation propensity, including: surface hydrophobicity, charge, and the propensity to form  $\beta$ -sheets and  $\alpha$ -helices.

Lazar, G. A., Dang, W., Karki, S., Vafa, O., Peng, J. S., Hyun, L.,...Dahiyat, B. I. (2006). Engineered antibody Fc variants with enhanced effector function. *Proceedings of the National Academy of Sciences*, 103, 4005-4010. doi: 10.1073/pnas.0508123103

- Covers the mechanism of action for mAbs, and discusses mutations made to the Fc to enhance effector mediated cytotoxicity.

Leader, B., Baca, Q. J., & Golan, D. E. (2008). Protein therapeutics: A summary and pharmacological classification. *Nature Reviews Drug Discovery*, 7, 21-39. doi: 10.1038/nrd2399

- Summarizes and categorizes more than 130 FDA approved therapeutic proteins, briefly reviews disease states, targets, and generalized mechanism of action.

Lee, C. C., Perchiacca, J. M., & Tessier, P. M. (2013). Toward aggregation-resistant antibodies by design. *Trends in Biotechnology*, 31, 612-620. doi: 10.1016/j.tibtech.2013.07.002

- Review of efforts taken to engineer reduced aggregation prone mAbs, heavy focus on molecular modeling.
- Discusses the general stability of each domain of the mAb and new advances in mutation algorithms to reduce aggregation while minimizing effect on antigen binding domain.

Lu, Z. J., Deng, S. J., Huang, D. G., He, Y., Lei, M., Zhou, L., & Jin, P. (2012). Frontier of therapeutic antibody discovery: The challenges and how to face them. *World Journal of Biological chemistry*, 3(12), 187-196. doi: 10.4331/wjbc.v3.i12.187

- Review of the technological state of the art with respect to antibody based protein therapeutics.
- Covers the current state of antibody discovery and development, the challenges faced in clinical development, and how some of those challenges are addressed.

Mahler, H.C., Friess, W., Grauschopf, U., & Kiese, S. Protein aggregation: Pathways, induction factors and analysis. *Journal of Pharmaceutical Sciences*, 98, 2909-2934. doi: 10.1002/jps.21566

- Identifies multiple stress factors involved in aggregation, and a discussion of pathway sensitivity toward these stresses.

Manning, M. C., Chou, D. K., Murphy, B. M., Payne, R. W., & Katayama, D. S. (2010). Stability of protein pharmaceuticals: An update. *Pharmaceutical Research*, 27, 544-575. doi: 10.1007/s11095-009-0045-6

- Update to a widely referenced paper from 1989, discussing the myriad physical and chemical forms of protein instability, and potential causation.

Reichert, J. M. (2013). Which are the antibodies to watch in 2013? *MAbs*, 5(1), 1-4. doi: 10.4161/mabs.22976

- Reviews the newest antibody based drugs, their targets, limited discussion of mechanism of action, and continued importance of their contribution to the current state of medical technology.

Roberts, C. J. (2007). Non-Native protein aggregation kinetics. *Biotechnology and Bioengineering*, 98, 927-938. doi: 10.1002/bit.21627

- Extensive review of the non-native aggregation pathway, and the current understanding of the kinetics governing this pathway.

Roberts, C. J. (2014). Therapeutic protein aggregation: Mechanisms, design, and control. *Trends in Biotechnology*, 32, 372-380. doi: 10.1016/j.tibtech.2014.05.005

- Excellent review of protein aggregation, focusing on both pathways, and the variables that seem to convey aggregation propensity.

- Discusses the current usage of various assays to monitor both aggregation pathways, and total aggregation, and the emerging technology involved in aggregation modeling, and its importance in mutagenesis.

Shi, S., Semple, A., Cheung, J., & Shameem, M. (2013). DSF method optimization and its application in predicting protein thermal aggregation kinetics. *Journal of Pharmaceutical Sciences*, 108, 2471-2483. doi: 10.1002/jps.23633

- In depth assessment of DSF as a tool to evaluate protein conformational stability, in comparison to more traditional assays.
- Identified areas of correlation between DSF and DSC, while reporting the incompatibility of the DSF assay with surfactants, a common excipient used in formulation design.

Tessier, P. M., Wu, J., & Dickinson, C. D. (2014). Emerging methods for identifying monoclonal antibodies with low propensity to self-associate during the early discovery process. *Expert Opinion in Drug Delivery*, 11, 461-465. doi: 10.1517/17425247.2014.876989

- Reviews alternate methods for aggregation monitoring in a screening process, as well as the unique stability demands on protein therapeutics as a function of their use.

Voynov, V., Chennamsetty, N., Kayser, V., Helk, B., & Trout, B. L. (2009). Predictive tools for stabilization of therapeutic proteins. *MAbs*, 1, 580-582. doi: 10.1073/pnas.0904191106

- Presents a molecular modeling tool called Spatial Aggregation Propensity to identify protein surface characteristics that are aggregation prone.

Yamniuk, A. P., Ditto, N., Patel, M., Dai, J., Sejwal, P., Stetsko, P., & Doyle, M.L. (2013). Application of a kosmotrope-based solubility assay to multiple protein therapeutic classes indicates broad use as a high-throughput screen for protein therapeutic aggregation propensity. *Journal of Pharmaceutical Science*, 102, 2424-2439. doi: 10.1002/jps.23618

- Establishes the ammonium sulfate based solubility assay as an effective method for monitoring native aggregation propensity. Suggests the broad applicability of this assay for a variety of protein classes, and how the environment impacts aggregation profile.
- Clearly reviews the method used to employ the KBS assay for bench scale high-throughput protein evaluation.

Zhang, A., Singh, S. K., Shirts, M. R., Kumar, S., Fernandez, E. J. (2012). Distinct aggregation mechanisms of monoclonal antibody under thermal and freeze-thaw stresses revealed by hydrogen exchange. *Pharmaceutical Research*, 29, 236-250. doi: 10.1007/s11095-011-0538-y

- High resolution analysis of the effects of stress on the aggregation propensity of a single mAb, suggesting the importance of CDR loops in the aggregation propensity of a mAb.
- Suggests that the effect of freeze-thaw on a mAb's aggregation propensity is native state induced, through a structural study of the aggregates formed.