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AGGREGATION, STABILITY, AND FORMULATION OF HUMAN ANTIBODY THERAPEUTICS

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Abstract

sp0020 Many human monoclonal antibodies display poor biophysical properties, such as low stability and a propensity to aggregate. These unfavorable tendencies can be even more pronounced for human antibody fragments, which often require a considerable degree of optimization. In this review, we describe methods for analyzing aggregation and stability of human antibodies and antibody fragments. We also provide an overview of recent approaches to improve these properties through engineering and formulation.

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I. Introduction

p0005 Therapeutic monoclonal antibodies (mAbs) are among the fastest growing class of drugs in the pharmaceutical sector. Between 1985 and 2008, several hundred mAbs have entered clinical development, including 147 human and 167 humanized monoclonals (Nelson et al., 2010). There are now often multiple competing products for given targets and diseases. For instance, there are currently four FDA-approved antibody therapeutics that target antitumor necrosis factor alpha (TNF-alpha). The rise in the commercialization of mAb therapeutics has been underpinned by the growth in the technologies and scientific understanding of their manufacture and formulation.

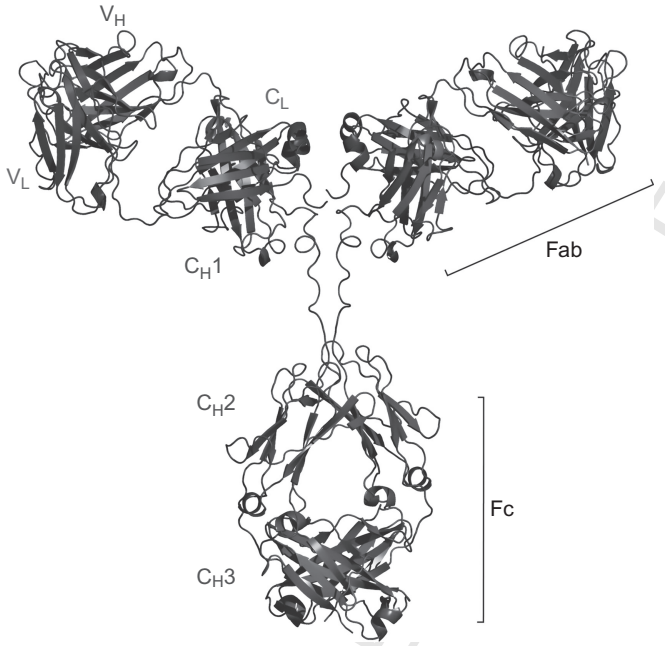
p0010 Antibodies have evolved to withstand the physiological stresses within the mammalian immune and circulatory systems and are found at milligram per milliliter concentrations *in vivo*. The recombinant manufacture of mAbs, however, subjects the immunoglobulin to various stresses, ranging from heat and pH to high concentration and mechanical strain (Lahlou et al., 2009). The final formulation of an mAb drug must be carefully chosen to allow drug manufacture at the appropriate scale and to optimize quality and stability of the final drug product. Moreover, there is an increasing clinical and commercial need for patients to self-administer the drug through subcutaneous delivery. This delivery route places additional requirements on the development and manufacture of mAb drugs, as it requires high-concentration liquid formulations of more than 100 mg ml^{-1} (Shire et al., 2004). For liquid preparations, viscosity must be minimized to facilitate sterile filtration prior to final filling. Further, low viscosity is required for maintaining accuracy of filling for prefilled syringes and to minimizing discomfort to the patient during administration. In addition, mAb therapeutics have to withstand storage for up to several years. This is necessary to manage product inventory and to minimize manufacturing campaigns. For storage, the protein is often frozen out of solution in high-volume vessels (Singh et al., 2009), which can result in changes in protein conformation and aggregation (Schwegman et al., 2009).

p0015 For each of the steps of expression, purification, concentration, formulation, storage, and final filling of the mAb, protein degradation, such as aggregation, must be minimized. Reduction of aggregation, in particular,

is vitally important, as it can lead to lower *in vivo* efficacy, increased variability among batches of the therapeutic, and perhaps most importantly, immunogenicity in patients (Cordoba-Rodriguez, 2008). The most commonly used mechanism currently used to reduce this is by controlling the formulation of the mAb, by optimizing solution conditions such as ionic strength, pH, and the addition of excipients (Wang, 1999). Optimization of each of these parameters can be challenging, however, due to interactions between them. Traditionally, generation of the optimal formulation conditions has been determined using long-term stability studies, whereby the mAb is incubated in various combinations of solution conditions under several environmental stresses, for example, temperature, followed by biophysical analyses to determine changes to protein stability. To reduce the time and resources taken to carry out such studies, efforts are underway to develop higher-throughput techniques to undertake formulation screening (Capelle et al., 2007; Goldberg et al., 2010).

p0020 Antibodies are large multidomain proteins, and factors that contribute to their stability and propensity to aggregate are complex and generally not fully understood. While formulation has some impact, clearly a vital consideration is the primary sequence of the protein itself. Although the modular nature of mAbs is such that the Fc region (and majority of the molecule) is largely identical between antibodies of a particular isotype, the Fab region differs greatly (Fig. 1). Consequently, there are significant differences in stability and aggregation propensity between antibodies, related to Fab differences and the particular antigen specificity of the monoclonal.

p0025 An additional mechanism that may also contribute to mAb aggregation may be the result of posttranslational modifications to amino acids during expression, purification, or storage (Jenkins et al., 2008). In particular, methionine oxidation to methionine sulfoxide or methionine sulfone and the deamidation of asparagines to form aspartic acid or isoaspartic acid have been shown to play important roles in the degradation of therapeutic proteins that can result in loss of activity (Taggart et al., 2000). There may, however, be a role in such changes in the aggregation of the protein, due to changes in surface charge. However, the precise role of such chemical modifications in antibody aggregation remains elusive. Au2



f0005 FIG. 1. Structure of the human IgG molecule. The protein consists of two disulfide-bonded chains, heavy (shown in blue) and light chain (shown in red). The smaller light chain consists of a variable domain (V_L) and one constant domain (C_L), while the larger heavy chain consists of a variable domain (V_H) and three constant domains (C_H1, C_H2, and C_H3). Antigen-binding propensity of the IgG molecule is mediated by its Fab portion, consisting of a V_H/C_H1 and V_L/C_L heterodimer. The C-terminal Fc portion is formed by association of C_H2 and C_H3 domains and mediates immune effectors functions and serum half-life. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this chapter.)

s0010 ## II. Expression of Human Monoclonal Antibodies and Antibody Fragments

s0015 ### *A. Expression and Purification of IgG*

p0030 All the currently FDA-approved therapeutic mAbs are of the IgG isotype, a complex 150 kDa human glycoprotein (Fig. 1). The vast majority of commercially produced IgGs are expressed recombinantly in mammalian cell lines, such as variants of Chinese hamster ovary (CHO) and murine

lymphoid cell lines, such as NSO and SP2/0. There have been large increases in the productivity of these systems over the past decade, with production yields often exceeding 5 g l^{-1} (Shukla and Thömmes, 2009). Au3
These increases have largely been achieved through advances in two areas, namely, (a) the generation of new and highly productive cell lines and (b) a more detailed understanding of chemically defined media and appropriate feeding strategies that achieve a high cell density and sustained viability over the course of the bioreactor run. This wide range of detailed improvements is beyond the scope of this chapter, but a number of comprehensive reviews of this area are available (Birch and Racher, 2006; Kelley, 2009). During mammalian expression, folding and glycosylation of the IgG molecule takes place in the endoplasmic reticulum (ER) lumen. Misfolded proteins can accumulate as intracellular aggregates within the ER. Molecular chaperones such as heavy chain-binding protein (BiP) facilitate high-concentration folding by binding to unfolded protein chains and preventing aggregation. Chaperones such as calreticulin and calnexin also facilitate correct folding by binding transiently to newly synthesized glycoproteins, ensuring that only correctly folded proteins are released from the ER (Molinari and Helenius, 2000). Intracellular aggregation of the antibody molecule due to misfolding, or protein multimerization due to noncovalent interaction of different molecules, is therefore likely to initially decrease the yield of secreted IgG into the production medium. Screening of production cell lines for IgG expression level, for example, by ELISA, therefore represents an indirect screen for mAb aggregation at an early stage. This production cell line screening takes place in a fairly high-throughput manner (up to 384-well plate) and so can be used to filter out those antibody variants most prone to aggregation within the cellular milieu.

s0020 ### *B. Expression and Purification of Antibody Fragments*

p0035 Unlike IgG, human antibody fragments can often be expressed in prokaryotic expression systems, such as *Escherichia coli* (Skerra and Pluckthun, 1988a,b). Au4
One of the main issues affecting the production of antibody fragments in bacteria relates to the fact that human immunoglobulin domains generally require the formation of intradomain disulfide bonds for correct folding. This is not compatible with the reducing conditions found in the bacterial cytoplasm and often results in the

recombinant protein being deposited as inclusion bodies. Genetic approaches to engineer bacteria with less reducing conditions have been undertaken, but overall with limited success (Martin et al., 2006). Others have selected mutations in human antibody fragments that stabilize them under reducing conditions (Tanaka et al., 2003). However, more commonly, the inclusion bodies have to be resolubilized using chemical denaturants and the antibody fragments refolded *in vitro* under oxidizing conditions (Cabilly et al., 1984). Unfortunately, aggregation tends to limit refolding yields, and it is often necessary to carefully optimize refolding conditions for each particular protein (protein concentration, pH, temperature, redox conditions, excipients). This affects a wide range proteins including antibody fragments (Buchner et al., 1992a,b) and cytokines (Lee et al., 2010). Au5

p0040 Problems associated with intracellular expression can be overcome by expressing antibody fragments under oxidizing conditions in the periplasm of Gram-negative bacteria (Skerra and Pluckthun, 1988a,b). Periplasmic expression in *E. coli* relies on the genetic fusion of the protein of interest with N-terminal leader peptides. While historically leader sequences that direct proteins toward the Sec pathway have been utilized (such as ompA, phoA, or pelB) (Skerra and Pluckthun, 1988a,b; Power et al., 1992), more recently, other secretion pathways (such as SRP) have also been used (Steiner et al., 2006). The leader peptide directs the recombinant protein to the periplasmic compartment, where the peptide is cleaved by signal peptidase. Folding of antibody fragments in the periplasmic space is aided by its overall oxidizing nature, as well as by the presence of chaperones (Buchner et al., 1992a,b; Lilie et al., 1993). However, both recombinant protein and endogenous protease concentration in the periplasmic space are high, which can lead to degradation of antibody fragments through aggregation or proteolysis. This particularly affects fragments with low stability or high-aggregation propensity, which may require further stabilizing mutations. For instance, it is generally not trivial to reformat IgG monoclonals into scFv, although they can often be expressed in a Fab format (Rothlisberger et al., 2005). Purification from the periplasmic space is straightforward, as the outer membrane of Gram-negative bacteria can be readily removed through osmotic shock conditions. Alternatively, the antibody fragments can be isolated from culture supernatant. In fact, after longer incubation times (more than 12 h), a considerable proportion of the fragments tend to leak from the periplasmic space into the culture medium. Similarly, antibody fragments can be

secreted into culture medium using recombinant expression in yeasts (such as *Pichia pastoris* or *Kluyveromyces lactis*). Expression in yeast often provides solid production yields, but variable posttranslational modifications (N-terminal processing, glycosylation) can occur and should be carefully monitored (Miller et al., 2005).

p0045 From culture supernatant or periplasmic preparations, antibody fragments can be purified by ion-exchange chromatography and immobilized metal ion affinity chromatography (IMAC) or alternatively through a single-step affinity chromatography step (Lee et al., 2007). For this purpose, protein A sepharose or protein L sepharose can be utilized, which bind to V_H3 and kappa variable domains, respectively.

s0025 III. Methods for Analyzing Solubility, Aggregation and Stability of Monoclonal Antibodies

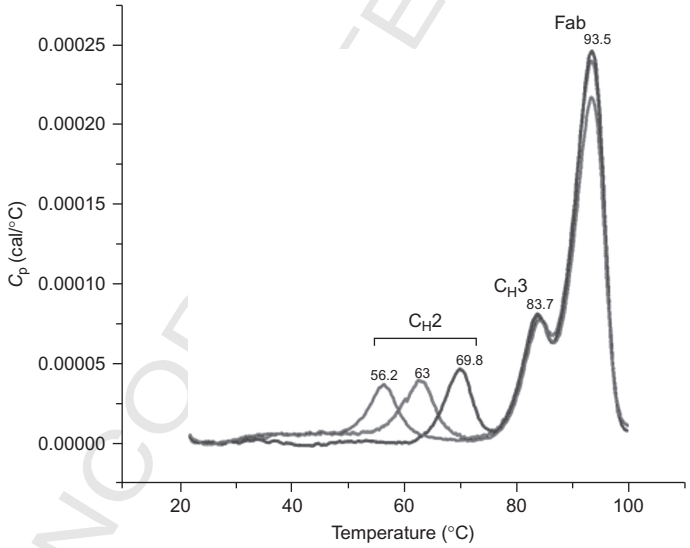
p0050 Protein stability can be considered a combination of conformational (thermodynamic) stability and colloidal stability (Goldberg et al., 2010). There are well-established assay systems available to measure both types of stability, although higher throughput and less sample-intensive assays are continually being sought.

s0030 A. *Differential Scanning Calorimetry*

p0055 Conformational stability represents the degree of unfolding and refolding of the native state and can be influenced by factors such as pH, ionic strength, and temperature (Wang, 2005). The most established method for measuring the thermodynamic stability of mAbs is differential scanning calorimetry (DSC). DSC instruments measure the heat capacity of the antibody solution as a function of temperature (Jelesarov and Bosshard, 1999). The DSC instrument is based on two cells, one containing a solution of the protein of interest and another containing the solvent alone. The cells are heated at a constant rate, typically at approximately $1\,K\,min^{-1}$. As the heat capacities of the solution of the protein and the solvent alone are different, a certain amount of electrical power is required to zero the temperature difference between the two. The power difference ($J\,s^{-1}$), after normalization by the scanning rate ($K\,s^{-1}$), is a direct measure of the heat capacity difference between the solution and the solvent. By calculating and plotting changes in the heat capacity of the

protein solution against the change in temperature, a distinct trace, referred to as a thermogram, can be generated (Fig. 2). As each domain of the protein is denatured, a peak is formed, as the denatured protein has a larger heat capacity than the folded, native form. When analyzed using DSC, mAbs have distinct profiles as each of the CH2, CH3, and Fab domains unfold. Antibodies with different degrees of conformational stability will thus give different profiles, due to difference in the temperature at which the different domains unfold (Ionescu et al., 2008). DSC therefore gives extremely precise data on the conformational stability of each of the domains of a particular antibody and allows for easy comparison of the conformational stability of multiple antibodies. A disadvantage of DSC, however, is that it takes significant amounts of time to run an experiment (several hours), and that large volume of material can be required for the analysis.

p0060 A number of alternate, higher-throughput methods have recently been developed as an alternative to DSC based upon fluorescent probes, such as anilinonaphthalene-8-sulfonate and SYPRO® Orange (Invitrogen, Carlsbad, California) that specifically bind to hydrophobic portions of proteins (Pantoliano et al., 2001; He et al., 2009; Goldberg et al., 2010).



f0010 FIG. 2. Differential scanning calorimetry (DSC) profiles. Shown are melting curves of three IgG variants with identical Fab portions, but different isotypes. The isotype variants differ in the melting temperature of their C_H2 domains.

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The thermal stability of a given protein can thus be monitored by tracing the fluorescence change as the probe binds to the hydrophobic patches exposed as the protein unfolds. From the curve generated by the thermal unfolding, the midpoint of the unfolding transition (T_m) can be determined. The T_ms of different samples are then measured against each other and a standard, to determine whether they have increased (higher T_m) or decreased (lower T_m) conformational stability. The main advantages of this approach compared to DSC are that it is quicker, requires less material, and is genuinely high throughput, as it can be carried out in a thermocycler (He et al., 2009; Goldberg et al., 2010). Screening large numbers of antibody variants, that is, screening hybridoma supernatants or outputs from phage display selections for conformational stability, therefore becomes a viable proposition.

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B. Dynamic Light Scattering

p0065 In addition to conformational stability, colloidal stability of the antibody preparation is of extreme importance. The size and nature of protein aggregates can range extensively from, at one extreme, rapidly reversible noncovalent small oligomers, such as dimers, trimers, tetramers, etc., through large aggregates of either covalent or noncovalent oligomers, all the way up to irreversible visible particulates (Philo, 2006). The mechanisms by which aggregation of proteins occurs are complex and not fully understood, but typically the aggregates increase in size over time, becoming larger and less reversible. The detection of irreversible aggregates in a protein solution can be determined using a number of techniques, the most common of which are dynamic light scattering (DLS), turbidity analysis by optical density spectroscopy, size exclusion chromatography (SEC), and analytical ultracentrifugation (AUC).

p0070 DLS is a method of measuring fluctuations in light scattering intensity through a solution, over a period of time, generally between 100 ns and 10s of milliseconds and differs from ''classical'' light scattering techniques in that the intensity of the light scattering is not averaged over the time scale. The time scale of the scattering fluctuations is directly related to the translational diffusion coefficient of the scattering particles, which is directly related to the size of the particles, that is, larger particles produce stronger scattering signals. DLS has been shown to be particularly well suited for the detection of large aggregates within mAb solutions (Nobbmann et al., 2007). The limitations of DLS are that the resolution is typically not strong enough to detect small

oligomers of protein and that it is not designed to be set up for higher-throughput analysis of large numbers of antibody samples.

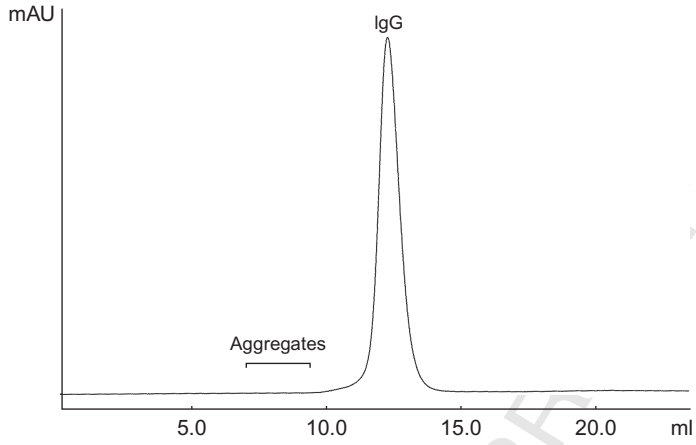
p0075 Recently, however, a higher-throughput alternative to DLS, called differential static light scattering (DSLS), has been described (Senisterra et al., 2006; Vedadi et al., 2006). The DSLS apparatus (Stargazer-384; Harbinger Biotechnology and engineering Corporation, Markham, Ontario, Canada) consists of a plate reader utilizing a peltier heating system and a CCD camera that allows 384-well measurement of light scattering and has been shown to rapidly detect aggregates in antibody samples in a comparable manner to DLS (Goldberg et al., 2010).

s0040 C. *Turbidity Analysis*

p0080 A simple measurement of protein aggregation is based upon optical density spectroscopy, whereby the optical density of protein samples can be measured in the near UV range (typically $OD_{350\ nm}$). As proteins aggregate and the solution becomes more turbid, the OD will increase. Various microtitre-based readers are available, some with in-built peltier heating systems, to allow the measurement of turbidity with increase in sample temperature. Turbidity measurement is fairly high throughput and thus is well suited for early analysis of antibody stability and, for example, the effects of mutations on aggregation propensity (Harn et al., 2007; Chennamsetty et al., 2009; Dimasi et al., 2009). The obvious limitation of using turbidity of a protein sample as a measure of stability is that only visible particulates are detected. It must therefore be used in combination with assays with a greater dynamic range in their ability to detect smaller oligomeric aggregates.

s0045 D. *Size Exclusion Chromatography*

p0085 SEC is the most widely used analytical system for determining the composition of protein samples (Fig. 3), due to the robustness of the Au6 data, the wide range of both high performance liquid chromatography (HPLC) systems and SEC columns that are commercially available and the relatively rapid generation of data (Arakawa et al., 2010). Protein samples such as antibodies can be separated such that both higher (oligomers, aggregates) and lower order species (unpaired chains, fragments, etc.) can be readily detected. There are many published examples of the use of SEC



f0015 FIG. 3. Size exclusion chromatography (SEC). Shown is a typical trace for an IgG monoclonal. The sample was analyzed on a Superdex 200 column (GE Healthcare).

for the characterization of mAbs and detection of aggregates, as well as the effects of changing buffer composition, pH, use of excipients, and mutations to the antibody sequence (Paborji et al., 1994; Usami et al., 1997; Andya et al., 2003; Pepinsky et al., 2010). Although a very robust system, some of the drawbacks of SEC include the trade-off between the dynamic range of analysis and the resolution possible, the potential for large aggregates to be physically filtered out of the sample, either via the column frits or interaction with the column bed and the possibility of interaction of the protein sample with the chromatography resin (Arakawa et al., 2010). Additionally, when analyzing by SEC, protein samples are often diluted into a solvent that can be quite different from the final formulation that has been chosen for production and storage. Care therefore needs to be taken when interpreting data and in choosing the most appropriate chromatography matrix, the correct solvent (mobile phase), and the use of appropriate known standard samples.

s0050 *E. Analytical Ultracentrifugation*

p0090 Although first developed in the early part of the twentieth century, AUC has recently received much interest as a tool for the analysis of protein aggregates, particularly with the generation of advances in computing

technology to aid in the analysis (Berkowitz, 2006). In contrast to SEC, AUC can generate protein size distribution data and sedimentation coefficients with minimal disruption to the protein oligomers themselves and importantly can be run at very high concentrations of the antibody directly in the formulation buffer. Thus, it is perhaps the least artificial system for analyzing size distribution in a high-concentration protein solution. The most widely used AUC method for analyzing aggregation is sedimentation velocity AUC (SV-AUC). This method involves accelerating samples to a high speed to generate a series of concentration profiles representing the migration patterns of all of the different macromolecular species in the sample. Software has been developed that can analyze the sedimentation coefficients generated and accurately determine the sizes of the components in the mixture. There have been several examples of the application of this technology to identify aggregates in mAbs (Franey et al., 2010; Gabrielson et al., 2010; Pepinsky et al., 2010). The limitations of this technology are predominantly the time and low-throughput nature of the analysis as well as the high cost of the AUC equipment itself. Nevertheless, it has been shown to generate very accurate aggregation data, particularly at the high concentrations of sample typically being used for mAb formulation.

p0095 The aggregation analyses described above are typically carried out on mAb samples during the formulation development phase, although, as previously described, efforts are underway throughout the biopharmaceutical industry to determine propensity to aggregate as early in the antibody discovery process as possible, where samples will be greater in number, but lower in concentration than in the final product formulation. For all new higher-throughput methods of analysis of both conformational and colloidal stability, care will need to be taken to ensure that results obtained at lower concentrations translate to those seen at the higher-concentration formulations used during manufacture and storage.

s0055 *F. Accelerated Stability Studies*

p0100 Therapeutic mAbs are typically stored over several months between manufacture, filling, and clinical administration. As detailed previously, they are typically formulated at more than 50 mg ml^{-1}, with subcutaneous

dosing requiring concentrations exceeding 100 mg ml^{-1}. To understand the stability and aggregation propensity of a given mAb in a given formulation, accelerated stability studies are performed during development. This involves the incubation of the antibody formulation at defined temperatures, typically 4 and 40 °C over several months. At defined interval, samples are removed and subjected to stability analyses, as described in the previous section. Predetermined limits of rates of aggregation as well as rate of appearance of lower molecular weight products of degradation over time are established and the antibody sample measured against, to determine whether the antibody is appropriately stable over long periods of time.

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G. In Silico *Screening*

p0105 Unlike biophysical methods, computational approaches have the advantage that they do not require protein expression and purification. However, most current computational methods for analysis of protein aggregation propensity are restricted to small proteins or structural motifs (Chiti et al., 2003; Fernandez-Escamilla et al., 2004; Tartaglia et al., 2005). The size and complexity of large molecules such as immunoglobulins has made the design of algorithms to predict aggregation propensity more difficult (Voynov et al., 2009). More recently, a promising predictive tool called ''spatial aggregation propensity'' (SAP) has been generated and shown to be able to predict regions of aggregation propensity on whole IgG molecules (Chennamsetty et al., 2009). This technology uses high-resolution atomistic computer simulations of the IgG molecule to assess each amino acid in terms of hydrophobicity, surface exposure and, importantly, the hydrophobic contributions of other amino acids within a predetermined radius. This generates a ''hydrophobicity heat map'' of the surface of the IgG molecule, allowing the identification of particular hydrophobic patches on the surface. The authors showed that replacing amino acids within these patches with more hydrophilic residues reduced experimental aggregation of the mAbs. Molecular simulation tools, such as SAP, have the potential to screen for monoclonals with reduced aggregation propensity, bypassing the need for laborious protein production and characterization. However, their impact on real-world applications remains to be determined.

s0065 IV. Addressing Aggregation and Increasing Stability of Monoclonal Antibodies

s0070 A. *Formulation*

p0110 The most commonly used mechanism to address mAb aggregation and to increase conformational and colloidal stability is to change the formulation of the product. This can involve multiple parameters, such as the ionic strength, pH, and the addition of excipients, such as amino acids, sugars, and detergents (Manning et al., 1995). Examples of particularly successful excipients are the amino acid arginine (Tsumoto et al., 2005) and the addition of sugars such as sucrose or trehalose (Andya et al., 2003). A therapeutic mAb will typically be prepared in a range of different formulations, with structural and colloidal stability analyses, as well as accelerated stability studies as described above, performed to explore the optimal combination of each parameter. These formulation studies can therefore be both time and product consuming, leading to a drive to develop higher-throughput methods for analysis.

s0075 B. *Isotype Switching*

p0115 Although broadly similar in structure, the IgG isotypes (IgG1, 2, 3, and 4) differ in structural detail (Padlan, 1994). Most therapeutic mAbs are either based upon IgG1 or IgG2, with no IgG3 and only a handful of IgG4 molecules in the clinic (Wang et al., 2007). There have been several studies comparing these isotypes in terms of stability and aggregation propensity. Results have been highly variable. For example, a recent study examined the stability of an anti-streptavidin mAb as IgG1 and IgG2 isotypes (Franey et al., 2010). This group showed that the IgG2 variant was more prone to aggregation. IgG2 molecules contain two additional disulphide bonds in the hinge region, compared to IgG1, which are capable of forming covalent intermolecular bonds between molecules. By contrast, another group investigated an anti-LINGO-1 antibody, using a range of both wild-type and mutated IgG1 and IgG2 variants (Pepinsky et al., 2010). They showed that there were both isotype-specific as well as Fab-specific factors contributing to aggregation propensity and that the wild-type IgG1 was less soluble than the IgG2. This work also

demonstrated that the different isotypes exhibited different aggregation mechanisms, both reversible and irreversible. Such studies indicate that isotype switching can be a useful tool to improve stability and reduce aggregation propensity but that, given the complex nature of IgG molecules, it is difficult to predict beforehand which will be most successful. For instance, IgG4-based molecules have recently been shown to exhibit Fab arm switching, which may affect their *in vivo* properties (van der Neut Kolfschoten et al., 2007).

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C. *Mutagenesis*

p0120 The Fab arm exchange observed in human IgG4 can be attributed to sequence motives in its constant domain hinge region (van der Neut Kolfschoten et al., 2007). It has been shown that mutagenesis of this region can be used to limit this effect and lock the IgG4 molecule in a monomeric state (Labrijn et al., 2009). Other mutations in constant domains have been reported that increase their stability or reduce aggregation (Teerinen et al., 2006). However, more often, the variation in the biophysical properties of human antibodies relates to their variable domains. This is a major problem in the development of IgG monoclonals, as it often cannot be overcome by isotype switching and formulation approaches. The problem is even more apparent for antibody fragments. This is probably not surprising as variable domains (as their name indicates) encode most variation in the antibody sequence. As the size of the protein decreases from IgG to Fab to scFv to antibody single domain (V_H or V_L), the proportion of variable sequence increases. In fact, in a human variable heavy domain (V_H) as many as a quarter of the residues may differ from one molecule to another. Moreover, single domains (and to some degree scFvs) lack the domain–domain interactions that stabilize larger antibody formats (Rothlisberger et al., 2005). This renders differences in their biophysical properties more apparent, which may be masked in a multidomain format. Consequently, many human antibody single domains display problems with solubility and aggregation.

p0125 Early approaches to improve the properties of human variable domains include the incorporation of mutations into framework regions. This work was inspired by the discovery of well-behaved variable heavy domains in camels and dromedars (Hamers-Casterman et al., 1993) and based on the

introduction of corresponding mutations (camelization) (Davies and Riechmann, 1994). However, while camelization did improve biophysics, it also affected chain pairing through mutation of the V_H–V_L interface (Riechmann, 1996a,b). In addition, it required mutation of highly conserved human framework residues, which has the unfortunate potential to generate new B- and T-cell epitopes. Au7

p0130 Elegant work by Ewert et al. (2003a,b) relied on structural information and human consensus sequences to characterize and improve human variable domains. They were able to demonstrate that certain human variable domain families (V_L and V_H1, V_H3, V_H5) had broadly favorable properties, while others were less stable and more aggregation prone. In particular, human V_H3 consensus domains were shown to have the most favorable properties among their human peers. V_H3 is the most commonly used domain family in the human antibody repertoire and also frequently found in human antibody therapeutics. However, the domains were still much more prone to aggregation than domains derived from camelids (Ewert et al., 2002). In particular, the V_H3 domains analyzed by Ewert et al. readily aggregated when heated above their melting temperature. Au8

p0135 More recently, human V_H3 domains that reversibly refold after heating have been reported (Jespers et al., 2004a). These domains were selected from repertoires that had undergone selection using ''heat-cool'' cycles on phage (Jespers et al., 2004b). The method is based on the fact that filamentous bacteriophage is remarkably resistant to denaturing conditions (such as heat or acid), allowing the displayed protein to be exposed to aggregation-promoting conditions. Antibody fragments that resist aggregation can then be isolated by affinity capture using a conformation-specific superantigen (such as protein A). The human domains selected on phage displayed highly desirable properties: they could be expressed at high levels in bacteria and did not stick to chromatography matrices. They were also generally resistant against other denaturants such as acid (Famm et al., 2008). However, their thermodynamic stability was not increased (Jespers et al., 2004b). Thus, reversible unfolding rather than increased stability seems to be the basis of their broad aggregation resistance. This is similar to what is observed for camelid domains (Ewert et al., 2002). It has been suggested that reversibility of unfolding may be particularly important in bacterial expression systems, which lack chaperones found in eukaryotes (Demarest and Glaser, 2008).

p0140 Although a single mutation (Ser35Gly) has been suggested as a main determinant of aggregation resistance of V_H domains (Jespers et al., 2004a; Barthelemy et al., 2008), the situation is clearly more complex. Indeed, as reported for other proteins (Chiti et al., 2003), hydrophobicity, beta-sheet propensity, and charge all seem to play a role (Dudgeon et al., 2009). However, the individual contributions of these propensities are currently unclear. These results further highlight the power of directed evolution, which allows the selection of molecules with desired properties (aggregation resistance), even in the absence of detailed mechanistic insights. Moreover, selection for aggregation resistance can be combined with antigen selections. Thus, work in our laboratory has demonstrated that high-affinity antigen binders can be selected from repertoires of aggregation resistant V_H domains (Christ et al., 2006, 2007). Such domains represent robust building blocks for mAb development and may lead to improved future generations of diagnostics and therapeutics (Demarest and Glaser, 2008).

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