FORMULATION AND PROCESS DEVELOPMENT STRATEGIES FOR MANUFACTURING BIOPHARMACEUTICALS

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Edited by

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A JOHN WILEY & SONS, INC., PUBLICATION

Published by John Wiley & Sons, Inc., Hoboken, New Jersey
Published simultaneously in Canada

Library of Congress Cataloging-in-Publication Data:

ISBN 978-0-470-11812-2

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

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DEVELOPMENT OF FORMULATIONS FOR THERAPEUTIC MONOCLONAL ANTIBODIES AND Fc FUSION PROTEINS

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16.1. INTRODUCTION

Monoclonal antibody-based therapies have become a huge area for biopharmaceutical development, with 18 monoclonal antibodies (Table 16.1) on the market and nearly 200 antibody molecules in clinical facilities [1–4]. Monoclonal antibodies for therapeutic and prophylactic indications over the years have moved from fully murine and humanized murine forms to completely human forms. There has also been a breakthroughs since the 1980s regarding purification [5], analytical methods including biological assays [6], and manufacturing aspects leading to the ability to prepare purer lots of monoclonal antibodies economically at large scales. The majority of monoclonal antibodies that are currently approved or in clinical development are focused on meeting therapeutic needs in the areas of oncology, autoimmune, and inflammatory diseases [1,4].

Antibodies and Fc fusion proteins are large macromolecules (typically >150 kDa), an order of magnitude larger than many other protein therapeutics such as cytokines, and are multidomain as well as typically glycosylated in nature, (if produced by mammalian cell culture [7]. Domains of antibodies have naturally evolved to associate with a variety of targets such as antigens and FcRn receptors with high

Formulation and Process Development Strategies for Manufacturing Biopharmaceuticals, edited by Jameel and Hershenson

TABLE 16.1. Detailed List of Antibody Products Approved and Marketed in the United States

Antibody Product	Manufacturing Company	Generic Name; Description	Delivery Route/ Dosage Form	Final Formulation Concentration,[a] mg/mL	Buffer Components; pH	Excipients
Avasatin®	Genentech	Bevacizumab; humanized IgG1, anti-VEGF	IV/liquid	25	Na phosphate; pH 6.4	Trehalose, polysorbate 20
Bexxar®	Corixam-GSK	Tositumomab-I131; murine IgG2a, anti-CD20, radiolabeled (^{131}I)	IV/liquid	14	Na phosphate; pH 7.2	NaCl, maltose
Campath®-1H	Millennium-ILEX	Alemtuzumab; humanized IgG1, anti-CD52	IV/liquid	30	Na,K-phosphate, pH 7	NaCl, KCl, NaEDTA, polysorbate 80
Erbitux®	Imclone Systems	Cetuximab; chimeric IgG1, anti-EGFR	IV/liquid	2	Na phosphate; pH 7.2	NaCl
Herceptin®	Genentech	Trastuzumab; humanized IgG1, anti-HER2	IV/lyophilized	21 (440 mg)	Histidine; pH 6	Trehalose, polysorbate 20, benzyl alcohol
Humira®	Abbott	Adalimumab; human IgG1, anti-TNFα	SC/liquid	50	Na phosphate, Na Citrate; pH 5.2	Mannitol, polysorbate 80
Mylotarg®	Wyeth	Gemtuzumab ozogamicin; humanized IgG4, anti-CD33, immunotoxin	IV/lyophilized	4 (5 mg)	Na phosphate	NaCl, sucrose, dextran 40
Othroclone OKT3®	J & J-Ortho Biotech	Muromonab-CD3; murine IgG2a, anti-CD3	IV/liquid	1	Na,K phosphate; pH 7	NaCl, polysorbate 80

Raptiva®	Genentech	Efalizumab; humanized IgG1, anti-CD11a	SC/lyophilized	100 (150 mg)	Histidine; pH 6.2	Sucrose, polysorbate 20
Remicade®	Centocorb	Infliximab; chimeric IgG1, anti-TNFα	IV/lyophilized	10 (100 mg)	Na phosphate; pH 7.2	Sucrose, polysorbate 80
ReoPro®	Centocor-Lilly	Abciximab; chimeric IgG1, anti-GPIIb/IIIa, Fab	IV/liquid	2	Na phosphate; pH 7.2	NaCl, polysorbate 80
Rituxan®	Idec-Genentech	Rituximab; chimeric IgG1, anti-CD20	IV/liquid	10	Na citrate; pH 6.5	NaCl, polysorbate 80
Simulect	Novartis	Basilixamab; chimeric IgG, anti-CD25	IV/lyophilized	4 (20 mg)	Na,K phosphate	NaCl, sucrose, mannitol, glycine; polysorbate 80
Synagis®	MedImmune	Palivizumab; humanized IgG1, anti-RSV	IM/liquid	100	Histidine	NaCl; glycine
Tysabri	Biogen Idec	Natalizumab; humanized IgG4, anti-4α-integrin	IV/liquid	20	Na phosphate; pH 6.1	NaCl; polysorbate 80
Vectibix	Amgen	Panitumumab; human IgG2, anti-EGFR	IV/liquid	20	Na acetate	NaCl
Xolair®	Genentech	Omalizumab; humanized IgG1, anti-IgE	SC/lyophilized	125 (202.5 mg)	Histidine	Sucrose, polysorbate 20
Zenapax®	Roche	Daclizumab; humanized IgG1, anti-CD25	IV/liquid	5	Na phosphate; pH 6.9	NaCl, polysorbate 80
Zevalin®	Biogen Idec	Ibritumomab tiuxetan; murine IgG1, anti-CD20; radiolabeled (^{90}Y or ^{111}In)	IV/liquid	1.6	Na acetate, Na,K-phosphate; pH 7	NaCl, human albumin

[a]From total lyophilized product if applicable.

affinity, which makes them useful protein therapeutics. In the body, antibodies typically have a half-life of 30 days. On the other hand, they are expected to be stable in storage for more than 2 years. The goal of a formulation program for therapeutic antibodies and Fc fusion proteins, as well as other protein therapeutics, is generally to develop a stable, robust formulation that minimizes physical and covalent degradation, ensures long-term storage stability, and prevents any adverse *in vivo* effects such as injection site, immunogenic or anaphylactic reactions. Additionally, instability of the antibody molecules can alter the pharmacology of the drug product as it affects both the pharmacokinetics in the serum and drug clearance from the body.

Antibodies and Fc fusion proteins, like other proteins, can be degraded under conditions where they are exposed to extremes of heat, freezing, light, pH, agitation, shear stress, metals, and substances such as silicone oil from prefilled syringes. Exposed surface residues of each antibody are unique and require specific formulation excipients to provide maximal stability against the aforementioned stresses. Assessment of the physicochemical and thermodynamic instability of antibodies using novel analytical technologies has led to the identification of several events that are more specific to the unique nature of this particular class of proteins such as variations in Fc glycosylation, Fc methionine oxidation, hinge region cleavage, and glycation of Lys residues [8]. An optimal formulation should minimize all such antibody degradation reactions in solution, or at minimum, mitigate those degradation reactions that impact critical quality attributes.

Biotechnology companies and contract research organizations are using improved analytical methodologies to monitor the degradation of the protein therapeutics during stability testing. Forced denaturation, agitation, and freeze–thaw studies are used to simulate the conventional stresses that a protein can undergo during production, shipping, storage, and administration. The effectiveness of forced degradation and stability studies done on a small scale to predict long-term, large-scale product stability depends on a number of factors: (1) the temperature dependence of the protein has to be understood; (2) accurate predictions of the shelf storage require that the protein system follow Arrhenius degradation kinetics over the temperature range that is used for the accelerated stability studies; and (3) the stability studies have to be conducted on multiple manufacturing lots that are representative of the commercial process.

One of the main challenges facing the manufacturers of biologicals in terms of formulation is demonstrating biocomparability in terms of product stability and clinical bioequivalence. The production of biological products is a complex process that undergoes continual development and refinement before commercialization and may continue post-launch. In most cases, any alterations in the process used to manufacture the antibody molecules can result in wide differences in the structural and functional properties of the molecules. These changes can alter the stability, clinical efficacy, and/or safety of the recombinant antibody therapeutic. Therefore, there is a need to perform formulation development studies on manufacturing lots that are representative of the commercial process for the approved and marketed drug product. Another issue is the complexity of *Escherichia coli* production processes for cytokines versus mammalian-cell-derived processes for antibody production, which results in heterogeneity in glycosylation patterns. Antibodies are often heterogeneous as a result

of charge variants, glycosylation differences, and disulfide chemistry. Formulation screening must be initiated early in development even before knowing the commercial drug dose and before the commercial process is set. The purity of the excipients used in the formulation may present an additional challenge. Vendor or lot differences in the purity of the excipients can jeopardize the consistency of the drug product. With all of these considerations, formulation development can be a considerable challenge. In addition, the formulation screens must be efficient to accommodate limited amounts of protein available for early formulation studies.

Regulatory agencies require rigorous testing procedures to determine the stability of a pharmaceutical formulation over time. Regulatory perspectives of the characterization and stability testing procedures have changed with advances in analytical technologies, especially in the fields of mass spectrometry and chromatography, and there is an increased understanding of the biology of recombinant proteins, as well as preclinical and clinical experience with many approved products. Also, there is a regulatory requirement to demonstrate that material or process changes during antibody production generate bioequivalent drug product.

16.2. MECHANISMS OF DEGRADATION

16.2.1. Physical Instability

16.2.1.1. Aggregation and Particle Formation. Aggregation and related particle formation is a dominant degradation pathway of antibodies and can occur during all stages of protein therapeutic processing and storage [3,9,10]. Aggregation of light-chain antibody fragments and their deposition into amorphous precipitates or insoluble fibrils has also been linked to amyloid diseases such as systemic amyloidosis [11–13]. Knowledge of the mechanisms underlying the protein aggregation processes is essential to develop rational *in vitro* preventive strategies. The aggregation phenomena can be stipulated by protein structural changes or by colloidal effects affecting protein–protein interactions [14]. Such events for proteins in general could occur via a simple diffusion-limited mechanism [15] or involve nucleation as the primary stage for further growth and propagation of aggregates [16,17]. From earlier studies it has become evident that proteins with dominating β-sheet content are prone to aggregation [18,19] and can self-assemble into either amorphous precipitates [15] or well-defined fibrils [17]. The aggregation process is also sensitive to a wide range of factors such as protein concentration, hydrophobicity, and charge as well as solution pH, ionic strength, and temperature [3,9,10,20]. Particle formation due to aggregation is a major issue, and control of particle levels for parenteral administration is necessary to prevent potential adverse reactions, as well as potential clogging of intravenous lines and filters. When high therapeutic doses are required, the need for high volumes may be countered by increasing the concentration of the antibody (sometimes several orders of magnitude higher than conventional protein therapeutics). This, in turn, may result in increased problems relating to aggregation and particulation.

Antibody aggregation is complex and can proceed through covalent or noncovalent association that is highly dependent on the solution conditions, including

pH, ionic strength, and excipients [21,22]. This association can be due to disulfide or nondisulfide covalent bonds, while the noncovalent associations can be due to hydrophobic or electrostatic interactions. Adding to the complexity, a given antibody can undergo multiple mechanisms of aggregation. Antibodies can undergo domain swapping, which can lead to altered structure and aggregation. Increasing the temperature and pH of the formulation often results in covalent crosslinking due to disulfide shuffling, while protein concentration, salt content, and other factors can promote non-covalent association. Antibodies have multiple intradomain as well as interdomain linkages through the disulfides [7], and these linkages have been found to undergo shuffling during processing leading to product heterogeneity and aggregation. Antibodies are also susceptible to photo-oxidation, which can lead to aggregation.

In most cases aggregation of protein molecules proceeds through a partially and reversibly unfolded conformational state that results from partial unfolding. This conformational state can be populated through the effects of solution conditions and the internal conformational stability of the molecule on the transition from native to unfolded states. A protein aggregate is formed between two or more molecules because of this higher-order structural disruption and exposure of hydrophobic regions leading to intermolecular interactions. This can eventually lead to aggregation and/or particulation [10,14].

After storage in solution under physiological conditions for a sufficiently long period, dimers may represent the main component of total aggregates [approximately 10%–30% (w/w) at a protein concentration of ~160 mg/mL] [23]. More recent studies have examined kinetic and thermodynamic aspects of the dimerization of IgG1s in solution [21,22]. Using a recombinant human monoclonal antibody that recognizes vascular endothelial growth factor (rhMAb-VEGF) as a model [21], it was found that aggregation rates were greater in slightly alkaline (pH 7.5–8.5) compared to slightly acidic (pH 6.5–7.5) conditions. A high-salt environment (1 M NaCl) also enhanced dimerization. The nature of the IgG1 dimers was found to be highly complex, resulting from different associations of the antibody domains [22]. In our laboratory, we have studied the structure, stability, and conformational dynamics of the Fab, Fab'_2, and Fc fragments of an IgG1 molecule [24]. Structural studies of the intact antibody and fragments showed that the structure of the Fc fragment is most susceptible to pH changes. Thermal, guanidine HCl–induced and urea unfolding studies at pH 7.4 and 5.0 showed differences in conformational stability of the various fragments at these two pH levels. Incubation studies performed with the intact protein and the fragments at 37°C and 50°C showed that the Fc fragment aggregated faster than did the Fab and the intact antibody. We proposed that Fc–Fc and possibly Fab–Fc are responsible for the aggregation and particle formation of the IgG1 antibody molecule as a result of temperature-induced stress. McAuley et al. [25] showed that disulfide bond formation in the human C_H3 domain plays an important role in antibody stability and dimerization. This domain contains a single buried, highly conserved disulfide bond. The authors showed that this disulfide bond significantly affects the stability and monomer–dimer equilibrium of the human C_H3 domain, which may have implications for the stability of the intact antibody.

Another study of empirical phase diagrams of monoclonal antibody solutions produced from spectroscopic data suggested (1) the existence of similar structural states

at low temperatures independent of concentration and (2) a decrease in the temperature at which phase changes were observed with increasing concentration. The decrease in structural stability observed in these studies is probably the result of aggregation or self-association of the recombinant MAbs on heating in crowded solutions, and not a decrease in the intrinsic structural stability of the MAbs [26]. A related investigation [27] found that at a given concentration, the phase separation temperature for proteins in general strongly increases with the molecular weight of the oligomers. These findings imply that for phase separation, the detailed changes of the surface properties of the proteins are less important than the purely steric effects of oligomerization.

During manufacturing or shipment, proteins endure high mechanical or shear stress through filtration, mixing, and agitation and are exposed to various interfaces. Partially denatured molecules expose hydrophobic regions within the molecule, which can then result in interaction, protein aggregation, and particle formation [9,28]. Antibodies, like other proteins, can interact with air–water interfaces and surfaces such as metals and other hydrophobic components. Prior to delivery to the patient, protein pharmaceuticals often come in contact with a variety of surfaces (e.g., syringes and stoppers), which are treated to facilitate processing or to inhibit protein binding. One such coating, silicone oil, has previously been implicated in the induction of protein aggregation [29].

16.2.2. Factors Affecting Physical Instability

16.2.2.1. Solution Conditions. Vermeer and Norde have noted that pH has a strong influence on the antibody aggregation rate [30]. Table 16.2 outlines typical antibody-related degradation reactions and their mediation through use of appropriate solution conditions such as pH. Proteins in general are often stable against aggregation over narrow pH ranges, and may aggregate rapidly in solutions at pH values outside these ranges (Fig. 16.1). Examples include low-molecular-weight urokinase [31], relaxin [32], recombinant human granulocyte colony-stimulating factor (rhGCSF) [33], and insulin [34]. Both pH and salt can play a very important role for antibodies in solution, as they control physical properties such as conformational and colloidal stability as well as the chemical stability of the protein. In our studies, we found that different domains of the antibody have different pH sensitivities. It has also been shown that low pH (pH 4–6) and appropriate salt concentration reduce aggregation of antibodies in solution by affecting the noncovalent interactions between the antibody molecules. The Fab fragment is most sensitive to heat treatment, whereas the Fc fragment is most sensitive to decreasing pH. The structural transitions observed by DSC and CD studies in the whole IgG is the sum effect of those determined for the isolated Fab and Fc fragments [30,35].

The total charge on the protein is affected by the solution pH and electrostatic interactions within the antibody molecules and with the ions. Electrostatic interactions can affect protein stability in different ways. The amino acids in the antibody can be charged with increasing acidity or basicity of the solution. This can happen at a pH away from the isoelectric point (pI) of a protein [36]. The increasing charge repulsion between these charged groups of the antibody in such a solution can destabilize the folded or native state because of the high charge density. Thus, pH-induced

TABLE 16.2. Typical Antibody-Related Degradation Reactions in Therapeutic Formulations

Degradation	Causes	Possible Solutions
Noncovalent aggregation	Structural changes, colloidal stability, heat and other physical stress, sorbitol crystallization during freezing	pH, buffer salt, ionic additives, protein concentration, improving raw-material purity
Covalent aggregation	Disulfide rearrangement	pH, prevent association
Isomerization	pH ~ 5	pH, magnesium chloride
Deamidation	pH<5 and pH>6	pH
Clip formation	Proteases, metals, impurity	pH, chelation of metals, purity
Oxidation	Free radicals, reactive oxygen, metals, impurities, hydrogen peroxide	pH, free-radical and reactive oxygen scavengers, metal chelation
Surface denaturation	Low antibody concentration, binding to surfaces, hydrophobicity	Surfactants, protein concentration, pH

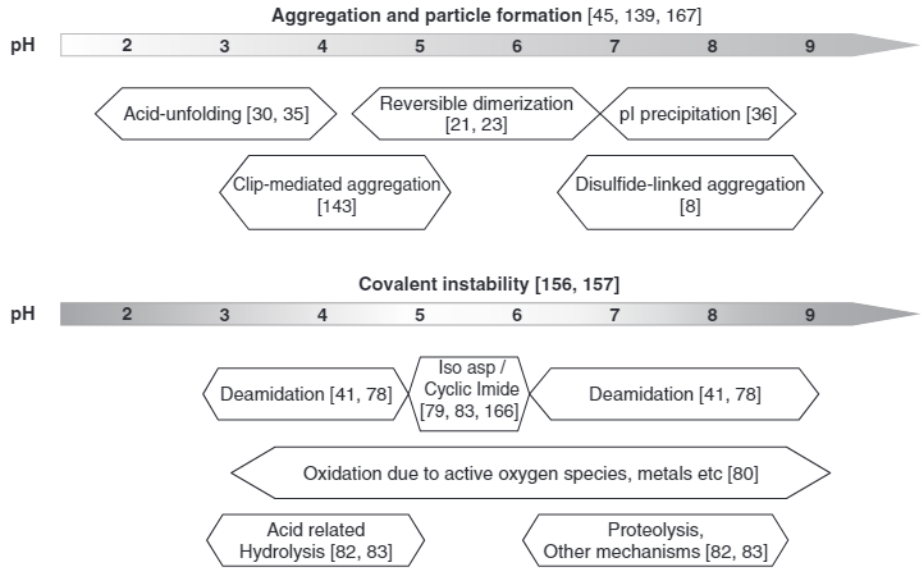

Figure 16.1. Different physical instabilities observed for antibodies at different pH levels along with some relevant references.

conformational unfolding can lead to a state of lower electrostatic free energy [14] Also, specific charge interactions, such as salt bridges, can affect antibody conformational stability. These salt bridges can stabilize the folded state and in some cases cause self-interaction [36,37]. When proteins possess both positively and negatively charged groups, the differential charge distribution on the surface of the antibody can cause protein–protein interactions, making assembly processes such as antibody aggregation energetically favorable [38].

16.2.2.2. Ligands and Cosolutes. Ligands and cosolutes are used in formulations to increase the physical stability of antibodies similar to other proteins. The Wyman linkage function applied by Timasheff [39] is commonly used to explain the effects of ligands and co-solutes in the formulation such as sucrose and salts. Through the Wyman linkage function, differential binding of ligand in two-state equilibrium will shift the equilibrium toward the state with the greatest binding. Binding of Zn^{2+} to human growth hormone or insulin is a very common example in which the free energy of unfolding for these proteins is increased and the native state of the protein is favored [40].

The Wyman linkage function can also be used to explain the effect of weakly interacting ligands (i.e., cosolutes), especially protein stabilizers such as sucrose and glycerol that are preferentially excluded from the surface of a protein molecule. In this case the degree of exclusion is proportional to the solvent-exposed surface area of the protein [39]. These cosolutes are excluded in the domain of the protein, and water takes its place in that domain, resulting in preferential hydration. Preferential exclusion can thus be interpreted as *negative* binding. During unfolding, protein surface area increases, leading to a greater degree of preferential exclusion. The net effect of greater negative binding to the unfolded state is to favor the native state.

Ligands and cosolutes that alter protein conformational stability also influence protein aggregate formation. For example, in the presence of polyanions, aggregation of acidic fibroblast growth factor [41] and native recombinant keratinocyte growth factor [42] are greatly inhibited. It has also been shown that the addition of weakly interacting, preferentially excluded solutes such as sucrose can inhibit aggregation of immunoglobulin light chains [17] and rhGCSF [15].

16.2.2.3. Salt Type and Concentration. Salts have complex effects on protein physical stability by modifying conformational stability and colloidal solubility, and may have different effects according to the surface charge of the protein or antibody. Salts bind to proteins, and destabilization of the protein can occur if the ions bind more strongly to the nonnative or unfolded state compared to the native state [43]. For example, the rate of aggregation of recombinant factor VIII SQ [44] was decreased in the presence of NaCl, while salt increased the aggregation rate for rhGCSF [33]. Moore et al. [21] found that salt increased dimer formation for IgG1 antibody.

Salts also modulate the strength of electrostatic interactions between the charged groups, at both intra and intermolecular levels. Thus, whereas intramolecular charge–charge interactions affect conformational stability, intermolecular electrostatic

interactions affect degradation rates. The overall effect of salt on protein stability is a fine balance of multiple mechanisms by which salt interacts with protein molecules and affects protein–protein interactions. Because pH determines the type, total, and distribution of charges in a protein, salt binding effects may be strongly pH-dependent. These results suggest that protein stability can be increased by improving the coulombic interactions among charged groups on the protein surface [37].

16.2.2.4. Preservatives. Antimicrobial preservatives, such as benzyl alcohol, are often utilized in liquid protein and antibody formulations to prevent bacterial growth during storage. In particular, multidose formulations of proteins require effective preservatives to prevent microbial growth after opening and administration of the first dose. Preservatives are also required for certain drug delivery systems. However, preservatives can interact with proteins and often induce aggregation of protein in aqueous solution. For example, preservatives (e.g., phenol, *m*-cresol, benzyl alcohol) have been shown to induce aggregation of recombinant interleukin-1 receptor [45] and recombinant human interferon gamma (rhIFNγ) [46].

Preservatives can bind to the nonnative or unfolded states and make the molecule prone to aggregation. For example, it was observed that addition of benzyl alcohol perturbed the tertiary structure of rhINFγ without affecting its secondary structure, and the rate of rhINFγ aggregation increased as the molar ratio of benzyl alcohol to protein increased [46]. Also, preservatives reduced the apparent melting temperature of recombinant interleukin-1 receptor [45].

16.2.2.5. Surfactants. Nonionic surfactants are often utilized in protein and antibody formulations to prevent aggregation, surface denaturation, and adsorption during purification, filtration, transportation, freeze drying, spray-drying, and storage. *Surfactants* (surface-active agents) are amphiphilic molecules that tend to orient such that the exposure of the hydrophobic portion to the aqueous solution is minimized. For example, surfactants adsorb at air–water interfaces, forming a surface layer of surfactant molecules oriented so that only their hydrophilic ends are exposed to water. Such orientation and surface adsorption can also occur at solid–water interfaces such as those found in vials, syringes, tubing, and other containers. Protein molecules are also surface-active and adsorb at interfaces. Surface tension forces at interfaces can perturb protein structure, often resulting in aggregation. Surfactants inhibit interface-induced aggregation by limiting the extent of protein adsorption [47].

As in other cosolutes, differential binding of surfactants to native and unfolded states of protein influences the protein's conformational stability. In some cases surfactants still can kinetically inhibit protein aggregation at interfaces despite causing a reduction in the thermodynamic stability of the protein conformation. This often helps prevent adsorption of antibodies formulated at low concentrations to interfaces such as IV lines, bags, and storage containers. In addition, surfactants have been shown to act as chemical chaperones, increasing rates of protein refolding and thus reducing aggregation [48,49].

16.2.2.6. Freeze–Thaw–Related Damage. Freeze–thawing is a common stress to which a therapeutic protein can be exposed to during manufacturing, shipping, and storage. Therapeutic proteins are purposely frozen for storage of bulk drug substance or for storage of analytical samples. The final commercial product also may be frozen accidentally because of mishandling. This process may happen once or multiple times, with additional damage to the protein potentially occurring during each subsequent freeze–thaw cycle.

Protein aggregation during freeze–thawing has been attributed to partial unfolding of protein molecules caused by the perturbing conditions arising during the process [50]. Perturbation of the protein conformation can be caused by low temperature [51], freeze concentration of solutes [52], pH changes due to buffer crystallization [53], exposure of protein molecules to the ice–liquid interface, and/or adsorption to the container surface [54,55]. Additionally, freezing-induced increases in salt concentration can reduce intermolecular repulsion (i.e., colloidal stability) between protein molecules via charge shielding, resulting in more favorable intermolecular interactions that lead to aggregation [14].

During freezing, there is also an increase in the concentration of protein molecules [56] when ice crystallizes and phase separates from the remaining amorphous material. Additional excipients (e.g., salt, mannitol) may also crystallize. In aqueous solution, increased protein concentration typically corresponds to an increase in the rate of aggregation. Although freeze concentration of a protein would therefore be expected to promote aggregation, it has often been observed that increasing the initial concentration of a protein will actually reduce the percentage of aggregation occurring during freeze–thawing [50]. It has been suggested that increasing the initial protein concentration reduces the fraction of protein molecules that is exposed to the ice–liquid interface, resulting in reduced aggregation. Thus, the effect of changing the initial protein concentration on damage during freeze–thawing can be difficult to predict.

Numerous factors can affect the magnitude and nature of freezing-induced stresses, as well as the protein's responses to them. Among the most critical are the pH and ionic strength of the solution, because these factors, in general, modulate both the conformational and colloidal stability of protein [14] as well as a protein's response to physical stresses. In addition, the warming and cooling rates used during freeze–thaw can alter the degree of macroscopic freeze concentration, surface area of the ice–liquid interface, and duration of exposure of the protein to these potential stresses. The container material, geometry, and volume can also affect protein damage during freeze–thawing by modulating the effects of adsorption of protein molecules at the liquid–container interface, and by altering cooling and warming rates.

In some cases additional changes in physical state have been observed during frozen storage. Piedmonte et al. [57] observed protein aggregation during storage of sorbitol-containing formulations at -30°C. The aggregation correlated with DSC melts that are characteristic of crystalline substances and suggest that the sorbitol crystallizes over time in the formulation. During freezing, the excipient must remain in the same phase as the protein to provide protein stability. By crystallizing, the sorbitol is phase-separated from the protein, which leads to protein aggregation.

16.2.2.7. Lyophilization-Induced Stresses. Typically, it is desirable to formulate therapeutic proteins in liquid formulations for ease of administration and lower cost of production. However, proteins in liquid formulations are generally at a greater risk of and physicochemical degradation. Liquid formulations are also less robust with respect to stresses experienced during shipping and handling. If the desired shelf life cannot be achieved in a liquid formulation, lyophilization is often the alternative. Lyophilized proteins are typically less susceptible to physicochemical degradation because of the scarcity of water and the greatly reduced mobility of molecules in the dried state [9,58]. Spray drying is also a potential technology for producing fine protein powders for inhalation drug delivery [59,60].

Although biopharmaceuticals are generally more stable in the dried state, it is well known that lyophilization and spray-drying processes themselves can be greatly damaging to proteins. Lyophilization involves two major steps; freezing of a protein solution and drying of the frozen matrix under vacuum. The freezing step can potentially destabilize or denature proteins by a variety of mechanisms, including cold denaturation, concentration and pH effects, and ice–liquid interfacial effects. The drying step can potentially damage proteins by disruption and/or removal of the hydrogen-bonding network of water molecules. These dehydration-induced stresses are also present during spray drying. Moreover, gas–liquid interface and exposure to high temperatures used during spray drying can induce additional damage [61].

The molecular mobility of amorphous pharmaceutical materials is known to be a key factor in determining their stability, reactivity, and physicochemical properties [62]. Molecular motions in amorphous systems are usually characterized by measuring the time dependence of some bulk property such as enthalpy or volume, or by using spectroscopic techniques to monitor the motions of particular functional groups. Usually such molecular mobility is quantified using relaxation time constants. Chang et al. [63] found that stability correlated best with the preservation of native structure for sucrose-based formulations, but with a trehalose-based formulation, neither structural relaxation time nor extent of native structure was predictive of stability. However, it is possible that the β relaxations rather than the α relaxation are critical to the stability. Plasticizers like glycerol may decrease τ for "α motion" but increase τ for "β motion" and stabilize proteins [64].

16.2.2.8. Interfacial Stresses. Many packaging components for parenteral products (e.g., glass syringes, vial stoppers) require the use of some form of lubrication for their processability and functionality. Siliconization of packaging components (such as glass, elastomeric closures, plastic, and metal) places a thin lubricating film on the surface of the components. Silicone oils have very low surface tension (20–25 mJ/m^2) and good wetting properties that permit the oil to spread readily on most solid surfaces [29,65,66]. Silicone oil is applied to cartridges and barrels of plastic and glass syringes to facilitate smooth and easy movement of plungers within the barrels. Further, silicone oil is applied to exterior surfaces of hypodermic needles to reduce the frictional drag and pain when the needles pass through the tissue [66]. Given the wide use of silicone oil coating on containers and closures, the potential for silicone oil-induced aggregation of therapeutic proteins is a major issue for product development.

Contamination of therapeutic protein formulations with silicone oil was first reported in the 1980s following observation of elevated blood glucose levels in patients administered with "cloudy" insulin from plastic syringes [65]. Analysis of the insulin formulations revealed that the silicone oil was causing protein particle formation. Although this problem has been longstanding, it has been described infrequently in the literature. More recently, Jones et al. [29] showed that silicone oil at a concentration of 0.5% (w/v) induced aggregation of four model proteins (ribonuclease A, lysozyme, bovine serum albumin, and concanavalin A) during heating. However, there is a lack of published reports on silicone oil effects on aggregation of monoclonal antibodies, for which hundreds of new products are in various stages of development and marketing.

It has been suggested that problems associated with silicone oil–containing syringes may easily be overcome by replacement with silicone oil–free syringes or by substituting silicone oil with other substances. Unfortunately, the suggested replacements for silicone oil such as Teflon® may also cause problems. It is important to recognize that the hydrophobic surfaces of Teflon have also been implicated in causing protein aggregation due to adsorption of protein molecules at the Teflon–solvent interface [67]. Therefore, although other alternatives to silicone oil are under exploration, work to understand how silicone oil induces protein aggregation is critical. This will be of great benefit in suggesting rationale and practical strategies to develop formulations that prevent this route of product degradation.

16.2.2.9. Consequences of Physical Instability of Antibodies on Safety, Efficacy, and Immunogenicity. The capacity of protein aggregates to enhance immune responses to the native form of the protein has been documented since the 1950s. Protein antigens presented in a highly arrayed structure, such as might be found in large native-like aggregate species, are highly potent in inducing immune response even in the absence of humoral immunity or T-cell help. It has been shown that the potency to induce immune response may be related to the ability of multivalent protein species capable of crosslinking B-cell receptor, which activates other immune pathways such as activation of B cells and class II major histocompatability factor (MHC) [68].

The formation of soluble aggregates in a protein formulation can have a significant effect on the pharmacokinetics and immunogenicity of the protein [68–71]. Insoluble aggregates or particles are considered undesirable for administration to patients. There are studies in the literature that directly correlate aggregate formation to loss of activity [72,73]. Several studies also show that administration of aggregated protein (e.g., aggregates of human serum albumin, human growth hormone) results in increased immune response and production of antibodies in the body against the therapeutic protein, which may further result in autoimmunity [68,74,75], that is, immune responses against the body's native protein. For some proteins, improper formulation or incompatibility of the protein with certain physiological conditions can cause injection-site reactions as well as formation of soluble aggregates at the site of injection [76,77]. The latter issue can lead to slow dissolution of the drug from the site of injection and thereby altered pharmacokinetics.

Thus, it is considered critical to minimize aggregation and particulation in the drug product.

16.2.3. Covalent Degradation

The covalent stability of antibodies is a complex function of solution conditions. Antibodies are known to undergo deamidation, isomerization, oxidation, proteolysis, and other covalent modifications, as a function of pH, temperature, and excipients. Table 16.2 outlines typical antibody-related degradation and its mediation through use of appropriate solution conditions. Deamidation has been identified as a source of charge heterogeneity of antibodies [41,78]. Deamidation can occur at asparagine or glutamine residues, resulting in a charge variant (aspartate or glutamate), isoform (cyclic imide or succinate), or cleavage product. Isomerization and cyclization can occur after an asparagine or aspartate residue, particularly if the subsequent residue is sterically small (e.g., glycine or serine). Both the charged isoforms and isoaspartate have been identified and characterized in antibody degradation studies [79]. Oxidation of methionine residues has also been observed in antibodies exposed to elevated temperature or intense light [80]. In addition to methionine, other residues that are sensitive to oxidation include cysteine, histidine, tryptophan, and tyrosine. Photooxidation of tryptophan, tyrosine, phenylalanine, and cysteines can lead to free-radical formation, crosslinking, and yellowing effects [81]. Proteolysis is another critical instability of antibodies that occurs in liquid formulation conditions [82,83]. Peptide mapping and mass spectral analysis can be utilized to detect and monitor a variety of covalent modifications in antibodies [84,85] as a function of formulation solution conditions [86,87]. In addition, disulfide bond scrambling, cleavages, covalent dimer formation, and other chemical or covalent degradation reactions have been observed in antibodies as a function of storage time. In liquid formulations, both IgG1 and IgG2 antibodies are prone to clipping via nonenzymatic fragmentation [83,88]. Site-specific fragmentation of the peptide backbone and disulfide bond linkage utilizing capillary electrophoresis and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has identified site-specific fragmentation of the peptide backbone and disulfide bond linkage. The resulting species include one-armed complexes missing one light chain or one Fab arm, in addition to free Fab or free heavy and light chain [82]. The cleavage sites are located predominantly in the hinge region of the heavy chain. The nature of the site-specific fragmentation in the case of hinge cleavage may be driven by molecular kinetics and not by protease contaminants [83].

16.3. METHODS

16.3.1. Physical Characterization

Physical characterization of the small, soluble aggregates of monoclonal antibodies has commonly been accomplished by established techniques such as size exclusion chromatography (SEC) [89–91] and gel electrophoresis [91,92]. Further characterization of antibodies has also been accomplished by orthogonal methods, including

laser light scattering [21,93], analytical ultracentrifugation [89–91], and field flow fractionation [91,94]. However, characterization of very large aggregates of monoclonal antibodies has been hampered by the lack of sufficient analytical methods that characterize small populations of subvisible and visible particulates well. Several techniques have been explored, including light obscuration [95], microscopy [96,97], electrical sensing zone instruments (Coulter counting) [98], dynamic light scattering [95], and turbidity/opalescence [95], although each has its own strengths and weaknesses. Aggregation propensity has been screened by examining thermal unfolding by differential scanning calorimetry [99–103] or Fourier transform infrared (FTIR) spectroscopy [101,102] and by determining second virial coefficients by techniques such as self-interaction chromatography [104] or light scattering [33].

Physical characterization of the formation of soluble aggregates such as dimers and small oligomers is commonly achieved by SEC. Gel electrophoresis is also used [92], but SEC has become the method of choice for high-throughput quantification of antibody aggregation. The molecular weight of early-eluting SEC peaks can be identified by online multiangle light scattering [21,93]. Light-scattering detection with SEC analysis also improves the ability to detect small amounts of large aggregates that may not have measurable UV signal, but because of their large size, scatter light [105]. Confirmation of the molecular weight by an orthogonal technique is important, as molecular mass standards can lead to erroneous results. For example, a dimer can be misidentified as a trimer [21], or there can be multiple peaks with the same molecular weight such as two-dimer peaks. There may even be an altered form or chemical modification to the monomer that elutes with a larger hydrodynamic radius [106].

However, it is also possible that sample preparation and SEC analysis itself may alter the distribution of monomeric and larger species. High-concentration antibodies are typically diluted prior to injection, which can allow dissociation of reversible aggregates. Analytical results would then vary depending on storage time and temperature between sample preparation and analysis. Methods where samples are injected without dilution can address these issues, but high-protein loads can lead to other problems. Injection into the mobile phase can also alter the aggregate content either by dissociation on dilution or by a change in solution conditions. Appropriate mobile-phase selection can also reduce column interactions resulting in very different elution profiles [107].

Sedimentation velocity analytical ultracentrifugation (SV-AUC) may be used to verify SEC analysis and characterize distributions in antibody formulations at 1–2 mg/mL up to possibly 10 mg/mL with minimal concerns over nonideality effects [89]. Measurements can be obtained at high concentrations (~40–50 mg/mL), but interpretation of the results is hindered by nonideal thermodynamic and hydrodynamic behavior [89]. The SV-AUC method can be used to aid in the selection of SEC mobile phases that give the most accurate aggregation results [89] and to quantitate aggregates present [108]. However, precision and accuracy of the aggregate levels determined by AUC is dependent on factors such as the quality of centerpieces used [108] and instrument condition [90].

The resolution of larger species by SEC is limited by column selection. For example, resolution on Tosoh Biosep G3000-type columns is limited to ~0.5 MDa

(trimer to tetramer) or ~20 nm. The upper limit on G4000-type columns is 7 MDa (<~50mer), with an approximate hydrodynamic diameter of 50 nm. Flow field flow fractionation techniques with light-scattering detection potentially increase the detectable size range of antibody aggregates to ~50 μm [90,94]; however, care must be taken to investigate the transition between normal and steric mode [94]. Both flow field flow fractionation (F4; also abbreviated FFFF) [90] and asymmetric flow field flow fractionation (AF4) [91,94,109] have been used to characterize antibody aggregation. Litzen et al. [109] demonstrated improved resolution of aggregate species by AF4 as compared to SEC. Gabrielson et al. [91] compared the levels of aggregate detected by SEC, AF4, and SV-AUC. In this study, SEC significantly underestimated the percent of soluble aggregate detected. Sedimentation velocity AUC detected the most, and AF4 detected slightly less soluble aggregate than did SV-AUC for unstressed samples. Dimer was purified by SEC and analyzed. Reanalysis by SEC within 1 h of collection showed reduced dimer with increased monomer and trimer. Sedimentation velocity AUC also revealed some polydispersity, although much less dramatic than the SEC results [91]. Both SV-AUC and AF4 are powerful orthogonal techniques in comparison to SEC, but require specialized training and instrumentation such that they are unlikely to replace SEC as the workhorse.

As aggregates become extremely large, they may also be detected by light obscuration techniques as described in USP<788> [110]. The USP method specifies the characterization of 10- and 25-μm particulates. Depending on instrumentation, light obscuration may also detect particulates as small as 1 μm and up to 400 μm. The USP limits for particulates were initially focused on foreign particulate matter and not specifically for protein or antibody solutions that may contain low levels of product-related proteinaceous particulates. The increased viscosities of high-concentration antibody formulations may also cause sample analysis issues [110].

Methods to characterize and monitor visible proteinaceous particulates are limited; USP788 describes a filter test to manually count all particulate matter collected on a filter after failure of the light obscuration test or if light obscuration is not suitable. Results from the filter method can differ significantly from light obscuration results, possibly because of the fragile nature of proteinaceous particulates. The filter method can also be used when visible particulates are observed. After particulates are collected on a filter, analysis may be performed to identify observed particulates by infrared spectroscopy or energy-dispersive spectroscopy. We used digital quantitative microscopy to determine the size distribution of particulate matter collected on a filter. A sample result is shown for stressed samples of a monoclonal antibody formulation with and without polysorbate 20 (Fig. 16.2). The addition of polysorbate reduces the number of particulates detected. A further modification to the USP technique was also reported, in which proteinaceous particulates were identified by staining with protein-specific dyes [96]. A drawback of such methods is that they are highly labor-intensive. Fluorescence microscopy after staining antibody solutions with Nile Red has also been used to detect particles ~1–10 μm in solution without filtration or dilution [111]. Automated methods for characterization of visible particulates are emerging, such as flow microscopy with digital image capture [97]. These methods store digital images for all particles detected, allowing further characterization of morphology

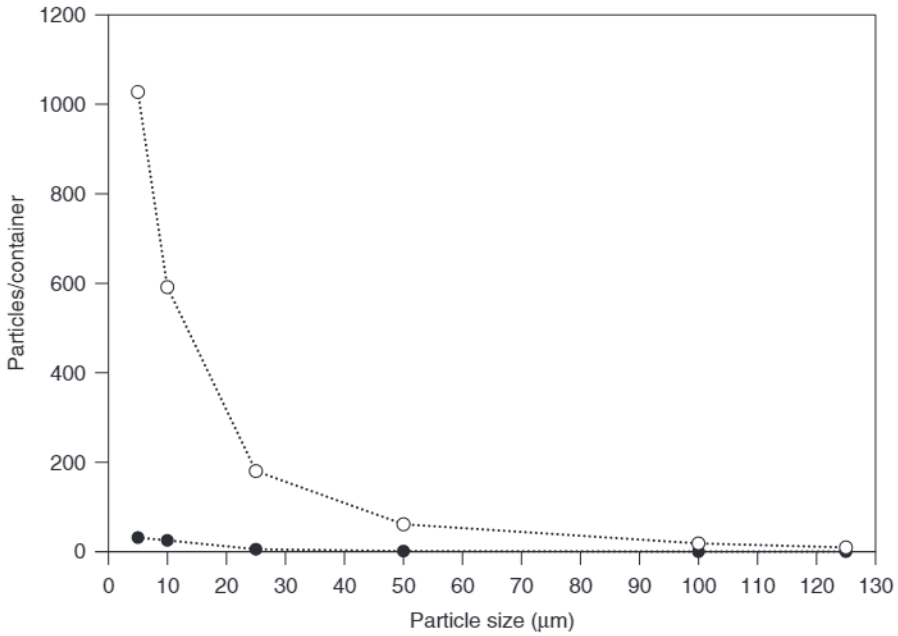

Figure 16.2. Particles counted as a function of size for a stressed monoclonal antibody formulation without polysorbate (○) and with polysorbate (●). Solutions were collected on a 0.2-μm white polycarbonate membrane filter. Particles with a dimension >10 μm were counted using a Clemex Vision PE system with a Nikon E600 microscope. Particle size is equivalent circular diameter. (Unpublished data.)

and size distributions, offering significant advantages over indirect detection by light obscuration [97]. This methodology is currently material- and labor-intensive, but is an excellent technique for orthogonal characterization of higher-throughput methods.

Multiple techniques are often necessary to characterize antibody aggregation. Demeule et al. [111] characterized antibody aggregates under two solution conditions (0.1% acetic acid with 50 mM magnesium chloride, and phosphate buffer) by light scattering, fluorescence microscopy, and AF4. Under stable conditions for the antibody no aggregates were observed by fluorescence microscopy with Nile Red, while AF4 showed a distribution of monomer (88%), low-molecular-weight aggregates (2%), and high-molecular-weight aggregate (10%) with a size $\sim 1.5 \times 10^6$ Da. Under unstable conditions, many spherical aggregates with a diameter ~3 μm were seen by fluorescence microscopy. These aggregates were fragile and easily disrupted by the crossflow during AF4 analysis. The large aggregates eluted in the steric mode under a mild separation method with diameter > 200 nm.

The presence of larger aggregates can also be monitored qualitatively by dynamic light scattering (DLS) [95,100,112] or opalescence/turbidity [87,95,112]. Kiese et al.

[113] monitored the timecourse of aggregation time by light obscuration, DLS, and turbidity of 2-mg/mL MAb samples subjected to mechanical stress (stirring or shaking). Increases in 10- and 25-μm subvisible particles were detected by light obscuration in samples subjected to mechanical stress. Dynamic light scattering also detected the presence of a second peak in some samples, although less reliably than light obscuration. Stressed samples became more turbid when monitored at 350 nm and 550 nm and by visual comparison with opalescent reference standards. Monitoring at 350 nm was found to be more sensitive than 550 nm, although both were qualitatively similar. Ahrer et al. [100] detected trace large aggregates by DLS and SEC with light scattering. Although DLS can be used to detect the presence of larger aggregates, care must be taken to avoid overinterpretation of results from heterogeneous samples.

Early formulation development requires assays that can quickly delineate more optimal solution conditions. Thermal stability is often used to try to predict physical stability [99,101,102,114] for long-term storage or physical stresses encountered during formulation and storage; DSC has historically been the method of choice [99]. More recently, FTIR has been compared to DSC [101,102] and evaluated as a way to predict stability at high concentrations [26,101].

Besides thermal stability, mechanical stress may also be used to evaluate aggregation propensity during formulation development. Mechanical stresses with air–liquid interfacial phenomena are frequently used [95,115], although the surface–liquid interface may also induce aggregation. Mahler et al. tested stirring in special cone-shaped vials and agitation by horizontal shaking [95], and found that stirring induced higher levels of smaller aggregates. Levine et al. [115] stressed samples by placing half-filled vials on an orbital shaker, maximizing the air–liquid interface. They also correlated lower-surface-tension measurements to increased susceptibility of monoclonal antibodies to precipitation.

Second virial coefficients (B22) can be used to determine solution conditions with increased protein solubility [116], and positive values have been correlated to decreased protein aggregation propensity [33]. Traditional methods to determine second virial coefficients, including static light scattering and membrane osmometry, are usually material- and labor-intensive [104,117]. Improved methods include self-interaction chromatography [104,118], dual-detector cells for light-scattering intensity and concentration by SEC [119] and composition-gradient light scattering [120]. The ultrasonic storage modulus was also found to correlate with second virial coefficients and can be used to monitor protein–protein interactions at high concentration [121]. However second virial coefficients do not always predict conditions of optimal formulation stability. In a study of IgG2 aggregation at 37°C, no correlation was found between aggregation and the second virial coefficient [119]. In fact, formulation conditions at pH 7.4 and 5.4 that did not show significant aggregation had more negative second virial coefficients than did those measured in pH 4.0 formulations, which did aggregate significantly. Interestingly, near-UV spectra did show differences as a function of pH that correlated with aggregation propensity.

16.3.2. Covalent Characterization

More recent advancements in analytical methodologies have advanced the covalent characterization of intact recombinant antibodies [122,123]. Typically, analytical methods such as cation exchange (CEX) chromatography, isoelectric focusing (IEF), capillary isoelectric focusing (CIEF), and capillary zone electrophoresis (CZE) are utilized to monitor charge variants of antibodies that arise from deamidation, glycosylation, and *C*-terminal lysine processing [79,124–131]. Likewise, hydrophobic interaction chromatography (HIC) is effective in separating oxidized or isomerized degradants [80,132]. Most of these analytical methods are incompatible with online mass spectral analysis because of the salts in the mobile phase. Under certain mobile-phase conditions, capillary electrophoresis can be utilized in tandem with mass spectrometry for identification of covalent heterogeneity and degradants [133].

Early attempts at reversed-phase analysis of intact antibodies had limited success in overcoming issues with poor recovery and column fouling [134]. Perfusion chromatography or HIC in reversed-phase mode have resolved light and heavy chains of antibodies, with insufficient separation capabilities for degradant species [131,134]. Analysis of antibody fragments and peptide maps by reversed-phase chromatography has been a reliable approach for full covalent characterization of antibodies, albeit time-consuming in sample preparation [131,134–138].

Dillon et al. have optimized reversed-phase chromatography conditions (Fig. 16.3) for intact recombinant antibody analysis with improved recovery, resolution, reproducibility, and column lifetime [122,123]. The key factors for improved chromatography are high column temperatures (70°C–80°C), mobile phases with high eluotropic strength solvents, and long alkyl chains of the stationary phase, such as the Zorbax stable bond [122] and Polaris ether column [123]. Reversed-phase analysis of intact antibodies is also capable of separating degradants such as cleavage products [122] and disulfide isoforms of the IgG2 subclass [123] (Fig. 16.3).

16.4. LIQUID FORMULATION STRATEGIES

16.4.1. Physical Stabilization

Several formulation papers report that antibodies in general are most stable at pH 4.5–5.5, and that aggregation increases dramatically near neutral pH or near the isoelectric point [21,103,131]. In certain cases, antibodies can form a reversible dimer under physiological solution conditions, and the self-association is driven by hydrophobic interactions [21]. In other circumstances, antibodies can self-associate to form primarily active covalent complexes [22]. The relative rates of clipping, irreversible aggregation, and reversible dimer formation are highly dependent on the solution pH and type of accelerated condition, such as elevated temperature or multiple freeze–thaw cycles [139]. However, in some cases antibody stability is not well correlated to protein concentration, buffer concentration, salt concentration, or agitation [139].

Physical stability of monoclonal antibodies can be affected by pH, and by the choice of buffer, stabilizers, and surfactants. To a lesser degree, selection of the buffer

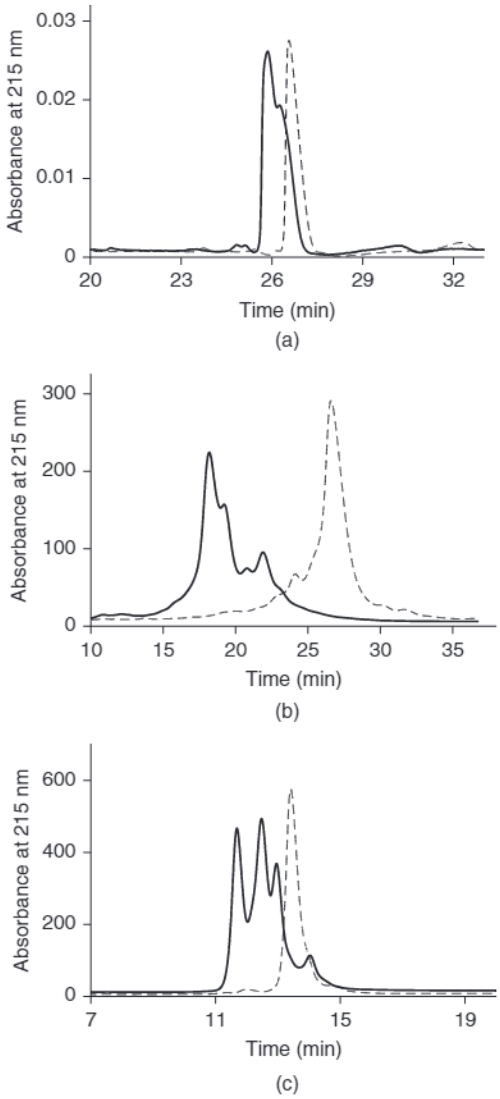

Figure 16.3. Analysis of an intact MAb expressed as an IgG1 (red) and IgG2 (blue) using (a) RP-HPLC, (b) CEX, and (c) CE-SDS. [More details are given in Wypych et al. [176] and Dillon et al. [123].

and buffer concentration at a given pH can also impact physical stability [140]. Stabilizing excipients include salts, polyols, and amino acids. Surfactants can minimize the formation of both soluble and insoluble aggregates due to surface interaction events, including agitation, and freeze–thaw. As concentrations of mAb formulations increase, drivers to identify formulations that optimize physical stability also increase because of concerns of potential immunogenicity of aggregates.

Criteria for the selection of pH include the pI of the MAb, structural stability, and sensitivity to other covalent reactions that could lead to aggregation. Formulating at a pH near the isoelectric point (pI) is undesirable as solubility may be reduced or opalescence/turbidity increased. If the pI of the antibody is near physiogical pH, formulation development activities may also need to include evaluation of physical behavior at physiological pH [141]. Although DSC can be used to explore the structural stability of monoclonal antibodies as a function of pH during preformulation studies, care must be taken in using the information as the primary criteria for pH selection, as thermodynamic stability does not always correlate with physical stability [114]. Covalent stability may also impact physical/structural stability. Covalent modifications could slightly alter the structural stability of the MAb and lead to aggregation [142]. Formation of clips due to acid hydrolysis can also lead to aggregation at elevated temperatures. Acid-induced clipping does not manifest at refrigerated temperatures; therefore, it is important to verify trends observed at high temperatures ($>37^{\circ}$C) [143].

Early monoclonal antibody therapeutics were often formulated near neutral pH at relatively low concentrations. More recently, antibody therapeutics with liquid formulations has been trending toward pH $\leq$ 6, and is provided at increasing concentrations. Many antibodies have optimal physical stability near pH 5.5. The dimer dissociation kinetics for an antibody to VEGF were examined between pH 6.5 and 8.5, and dimer formation was found to be slowest at pH 6.5, where dissociation was fastest [21], while insignificant dimer formation occurred at pH 5.5 at 30 mg/mL. Incubation of a chimeric antibody at 60°C resulted in minimal amounts of higher-molecular-weight species at pH $>$ 5.0, while freeze–thaw stability was optimal at pH $<$ 5.5 or pH $>$ 8.0 [139]. Significant precipitation was also observed in antibody solutions at pH $<$ 4 [86]. Selection of pH can be the most critical parameter during formulation development. For example, while aggregation was not significantly influenced by buffer concentration, antibody concentration, or sodium chloride concentration, the dominant factor was pH [139] at all temperatures studied (from 30°C to 50°C).

Once the optimal pH range has been determined, stabilizing excipients can be used to further minimize aggregation. Salts have historically been used as the primary choice for tonicity modifiers in MAb formulations; however, addition of sodium chloride at pH 7.5 actually increased the rate of dimer formation for MAb VEGF [21]. Polyols and sugars and more recently amino acids have been recognized as desirable stabilizing agents. Addition of sugars (lactose, sucrose, and mannitol) or amino acids (L-arginine, L-lysine, and L-alanine) prevented freeze–thaw-induced aggregation [139]. Histidine was also found to have cryoprotectant properties comparable to sucrose when used at concentrations above 50 mM [140]. At accelerated temperatures, histidine combined with arginine outperformed sucrose in preventing aggregation [140]. Histidine also minimized aggregation as compared to citrate and succinate at pH 6 [140]. Additionally, increasing concentrations of histidine reduced the viscosity of a 150-mg/mL antibody formulation. However, there are concerns with compatibility of high concentrations of histidine HCl with stainless-steel containers, as leaching of iron, with potential coloration of protein product due to degradation, is possible [140,144,145]. Addition of sodium chloride and other salts to high-concentration antibody formulations reduced the solution viscosity [146]. Addition of low levels

(0.1% and 1%) polyvinylpyrrolidone significantly reduced heat-induced aggregation. However, levels greater than 1% led to increased aggregation and even precipitation [147]. A formulation that was stable when stored at −20°C or subjected to multiple freeze–thaws was observed to aggregate on long-term storage at −30°C, which was attributed to crystallization of sorbitol [57].

Addition of surfactants to antibody formulations can minimize the formation of both soluble and insoluble aggregates. Surfactants primarily provide protection against surface denaturation phenomena that occur during freeze–thaw or agitation stresses. Polysorbate 80 and pluronic F68 effectively prevented antibody precipitation due to agitation stresses designed to simulate shipping, and performed better than did Brij 700, a less active surfactant [115]. Addition of 0.01% polysorbate 80 to 2-mg/mL antibody formulations in phosphate buffered saline effectively prevented formation of subvisible particles and minimized turbidity due to stirring or shaking [95]. However, surfactants may not always be effective at preventing freeze–thaw-induced aggregation, especially if the aggregation is not driven by surface phenomenon as seen where addition of polysorbate 80 did not prevent freeze–thaw-induced aggregation [139].

Although counterintuitive, increasing the protein concentration can actually help stabilize the formulation to surface denaturation-induced aggregation, as the ratio of protein on the surface to protein in solution is greater at lower concentrations [148,149]. Also, as antibody concentrations increase, the solutions become more opalescent, which can be an indicator of self-association. For an IgG1, this opalescent appearance was found to be a consequence of Rayleigh scattering and not self-association or physical instability at room temperature [112]. However, at 5°C, the turbidity was nonlinear with concentration and indicated possible reversible noncovalent association [112]. Opalescence may be a natural characteristic of high-concentration antibody formulations, but further work to determine effects of formulation conditions is necessary, particularly at refrigeration temperatures.

16.4.2. Container–Closure Issues

The impact of primary packaging components on stability should be considered during formulation development activities. The protein or an excipient could leach materials from the primary packaging system. Examples of leachables observed in therapeutic protein products that led to unanticipated degradation include metal ions or vulcanizing agents from rubber stoppers, barium from glass vials, and tungsten oxide from prefilled syringes [150]. These issues are not limited to the primary packaging, as sometimes even short-term exposure to materials such as silicone oil during administration with disposable syringes can also lead to protein instability [150]. Silicone oil exposure can lead to aggregation [29] and is commonly used in both disposable and prefilled syringes. Stoppers are often siliconized for machinability, but crosslinked siliconization coating processes can be utilized to minimize exposure of the protein to silicone oil and other agents in the rubber stoppers. Formulation development can address such problems, for example, addition of EDTA to prevent metalloprotease activation by metal ions leached from stoppers [150]. Plastic vials have also been examined as potential alternatives to traditional glass vials for protein therapeutic agents [151].

16.5. HIGH CONCENTRATION LIQUID STRATEGIES

16.5.1. Formulation Issues

Development of formulations for high-concentration protein drugs can be quite challenging. Some proteins pose outright solubility issues at higher concentrations. Even when solubility is not limiting, high-concentration formulations place additional challenges on other areas, including manufacturing, stability, analytical, and delivery issues. Concentration and concentration-dependent degradation routes, such as aggregation, and particle formation may become much more critical at higher concentrations. Also, the high viscosity of these formulations complicates injectability of the formulation [146]. Additional analytical techniques may be required to allow for direct measurement in the formulation without substantial dilution of the protein [38]. Operationally, solubility of proteins could be described by the maximum amount of protein in the presence of cosolutes whereby the solution remains visibly clear (i.e., does not show protein precipitates, crystals, or gels), or does not sediment at 30,000*g* centrifugation for 30 min [152]. The dependence of protein solubility on ionic strength, salt form, pH, temperature, and certain excipients has been mechanistically explained by changes in bulk water surface tension and protein binding to water and ions versus self-association. Net charge of the antibody is a major determinant of solubility [153]. Binding of proteins to specific excipients or salts influences solubility through changes in protein conformation or masking of certain amino acids involved in self-interaction.

Common measures to minimize aggregation of antibodies in high-concentration formulation include ensuring raw-materials purity and product homogeneity; finding the optimal pH range for maximizing physical stability; adding excipients such as sugars, ionic additives, amino acids, and surfactants; and finding the right protein concentration range required for delivery of therapeutic doses in acceptable volumes, with minimal degradation. The relationship between concentration and aggregate formation depends on the mechanism of aggregation. As the protein concentration increases, the fraction of the total volume occupied by the protein increases. The resulting decrease in the effective volume available to the protein yields a higher apparent protein concentration that, in turn, favors self-association. This nonideal behavior of increasing the apparent theormodynamic association constant with increasing protein concentration may be shelf-life-limiting, especially when the resulting aggregates are irreversible.

In some cases it may be possible to take advantage of the requirement for a high protein concentration. For example, Gokarn et al. [154] explored a novel approach of controlling the formulation pH by harnessing the ability of MAbs to "self-buffer." Buffer capacities of four representative IgG2 molecules (designated "MAb1 through MAb4") were measured in the pH 4–6 range. The buffer capacity results indicated that the MAbs possessed a significant amount of buffer capacity, which increased linearly with concentration. The long-term stability of the self-buffered liquid formulation was comparable to the conventionally buffered formulations. No significant change in pH was observed in the self-buffered formulation after 12 months of storage at 37°C

and 4°C. The 60-mg/mL self-buffered formulation was also observed to be stable to freeze–thaw cycling.

16.5.2. Viscosity

The majority of marketed MAb products are administered by intravenous infusion because of the requirement for high administered doses [155]. Although in certain indications this route of administration may be acceptable, in other cases it may present significant patient compliance, acceptability, and cost of treatment issues that render it undesirable. The limitations in the injection volumes required for subcutaneous (SC) administration (poor tolerance of volumes in excess of about 1.5 mL) and the large dose requirements of antibody treatments (in excess of 100 mg, typically 100–400 mg) necessitate their formulation as high-concentration solutions or suspensions. However, development of high-concentration protein formulations poses significant challenges, primarily because of their susceptibility toward concentration-dependent aggregation and their tendency to form viscous solutions, due to their high potential of intermolecular interactions and macromolecular crowding in solution. Macromolecular crowding (i.e., excluded volume effects) can impact protein physical properties such as viscosity, which can have a major impact on the ability to manufacture high-protein-concentration formulations as well as on the ability to administer the protein drug by injection. In general, the viscosity of a macromolecule in solution is dependent on interaction of two, three, or more protein molecules. In the case of significant protein self-association that results in formation of soluble aggregates, such interactions will lead to large increases in viscosity as a function of concentration.

These issues have motivated antibody innovators and drug delivery providers to evaluate strategies to enable SC delivery of MAbs. Some of these rely on improving solution stability and reducing the viscosity of the high-concentration protein formulations. Liu et al. [146] have proposed that manipulation of the solution conditions, such as ionic strength, buffer species, and pH, lead to a significant viscosity reduction of high-concentration solutions of recombinant anti-IgE MAb (rhuMAb E25 and E26).

16.5.3. Covalent Stabilization

Formulation approaches to covalent stabilization have been applied with moderate success. Often, the formulation pH is fixed according to solubility or aggregation constraints, and covalent stability may be compromised under such solution conditions [156]. If excipient strategies for covalent stabilization are insufficient, a lyophilized formulation may be a more effective approach to minimize covalent degradation under solution conditions constrained by physical stability requirements [157]. If no such constraints in physical stability or solubility exist, then the simplest approach to minimizing covalent degradation is to optimize the formulation pH. Oxidation, disulfide exchange, and deamidation can be minimized by lowering the pH. Conversely, increasing the pH can be an effective formulation strategy to control isomerization and hydrolysis, which are problematic under acidic solution conditions [9].

In addition to pH optimization, specific additives or formulation conditions have been found to be effective at controlling certain degradation reactions. General principles of formulation strategy for each specific mechanism of degradation are depicted in Figure 16.1. For oxidation issues, the addition of antioxidants, oxygen scavengers, or free-radical scavengers can inhibit degradation [80]. If the level of oxidizing agent is rate-limiting, free methionine in molar concentration exceeding that of the therapeutic antibody can serve as a scavenger to preferentially oxidize, instead of the antibody. Chelators, such as EDTA or citrate, may be effective at inhibiting metal-induced oxidation. A nitrogen overlay during drug product filling can prevent the oxidation that normally occurs by removing the oxygen in the air within the headspace that equilibrates with the drug product solution during the shelf life of the drug. Note that impurities in certain excipients, such as polysorbate [158] or benzyl alcohol [159,160], may contain free radicals from peroxides that can catalyze oxidation reactions, and proper sourcing and handling of these high-risk excipients can impact stability. Similarly, disulfide exchange reactions can be controlled by adding thiol scavengers and antioxidants or eliminating oxidizing excipients and trace-metal contaminants that can catalyze disulfide exchange [161,162]. Photooxidation is another related covalent degradation issue common to therapeutic antibodies [163]. Limiting UV exposure during purification and protecting the drug substance and drug product from light during storage, filling, distribution, and patient use help minimize photooxidation [81]. However, dark reactions continue to propagate after even brief exposure to intense light, such as that of automated visual inspection.

Deamidation is also a common covalent degradation mechanism of antibodies [41,78]. If pH reduction is ineffective or restricted because of other stability concerns, then buffer optimization may be an effective approach. Certain amine buffers, such as Tris, ammonium, or imidazole, can inhibit the deamidation reaction, and conversely, phosphate buffer can increase deamidation [164]. Excipients can also influence the rate of deamidation in both liquid and lyophilized states [165]. Under acidic formulation conditions, isomerization and hydrolysis at aspartic acid residues can occur [79,83], and the addition of magnesium has been found to inhibit the isomerization reaction [166]. Other forms of protease-mediated cleavage reactions can be inhibited by the addition of chelators to antagonize metalloproteases, protease inhibitors, pH optimization, and improved purification processes to remove the protease contaminants. Increased sucrose can also promote covalent stability by preferential hydration.

While liquid antibody formulations are less expensive, and generally easier to prepare for administration than are alternative formulation approaches, liquid antibody formulations are prone to oxidation [80], deamidation [41,78], aggregation [21,45,139,167], and fragmentation [82,83] as discussed above. In each of these events, water is the common culprit; water mediates electron transfer during oxidation and deamidation events and is also critical for fragmentation. The thermodynamic stresses that lead to protein aggregation result from exposure of hydrophobic protein surfaces to water and trying to find a lower-energy state by nonnative protein–protein interactions. Thus, removal of water can stabilize antibody-based drugs. Lyophilization or introduction into hydrophobic polymer systems can reduce the impact of water on antibody drug formulations.

16.6. LYOPHILIZED FORMULATION STRATEGIES

16.6.1. Stability and Reconstitution Issues of High-Concentration Lyophilized Formulations

Freeze drying or lyophilization is a widely used method for protein therapeutics to improve storage stability [9]. Lyophilization allows storage at high temperatures, and reduces conformational mobility due to slowed molecular motion resulting in decreased degradation of proteins. A lyophilized formulation may be the only practical choice for stabilizing many therapeutic proteins prone to degradation.

Antibodies may be damaged as a result of lyophilization as demonstrated by extensive formation of insoluble aggregates [54,149,168]. Improved native-like structure and a reduction in antibody aggregation can be obtained by incorporation of a carbohydrate excipient in sufficient quantities to fulfill the hydrogen-bonding requirements on the protein surface, suggesting a critical role for non-water molecules to act as replacements for water during drying and in the dry state [63,169–171]. Raman and FTIR spectroscopy have been used to monitor structural changes in the antibody in solid state [172].

The rate of rehydration during reconstitution of lyophilized formulations is a critical parameter. If the rate of rehydration is sufficiently slow to allow recovery of native conformation as water replaces nonwater excipient molecules, reconstitution will typically provide a satisfactory outcome for the antibody. If, however, the antibody does not have sufficient time or capacity to recover its native state during reconstitution, extensive aggregation can result. Excipients that have been reported to slow the reconstitution rate and thereby preventing such changes in the rehydrated antibody are glycerol [171] and other polymeric excipients.

Pharmaceutical scientists frequently use increased temperatures for accelerated formulation stability studies. Although such studies can be used to assess formulation parameters such as pH and ionic strength, antibodies lyophilized in the presence of a cryoprotective sugar excipient provide a more complicated picture. Lyophilized amorphous solids composed of a monoclonal antibody and a sugar cryoprotectant material such as sucrose or trehalose form glassy structures, liquids that are too viscous to flow. Antibody–excipient complexes exhibit a glass transition temperature (T_g), and materials have different properties above and below this value. To predict shelf life for accelerated degradation studies, it is important to use testing temperatures that do not exceed the T_g threshold of the complex or the collapse temperature. The apparent glass transition temperature of formulations in frozen condition is denoted as T_g'. Both T_g' and collapse temperature are close together for most proteins at low protein concentrations [58]; however, at higher protein concentrations, the difference between collapse temperature and T_g' are larger. Primary drying can be shortened by using conditions in which the product temperature substantially exceeds T_g' without any apparent detrimental effect to the product [172]. Annealing of rapidly cooled solutions results in significantly less aggregation in reconstituted freeze-dried solids than in nonannealed controls, with a corresponding decrease in specific surface area of the freeze-dried, annealed system [54]. Increased concentration of the IgG significantly improved the stability of the IgG against freeze-drying-induced aggregation, which may be explained

by a smaller percentage of the protein residing at the ice/freeze–concentrate interface as the IgG concentration was increased. Added salts such as NaCl or KCl contribute markedly to insoluble aggregate formation [54].

Sugars are commonly used as stabilizers in lyophilized protein and antibody formulations. Various molar ratios of sugar to protein have been tested, and the stability of the resulting lyophilized formulations has been determined by measuring aggregation, deamidation, and oxidation of the reconstituted protein and IR spectroscopy (secondary structure) of the dried protein. In one case a 360:1 molar ratio of lyoprotectant to protein was required for storage stability of the protein, resulting in a sugar concentration three- to four-fold below the isoosmotic concentration. Formulations with combinations of sucrose (20 mM) or trehalose (20 mM) and mannitol (40 mM) had stability comparable to those with sucrose or trehalose alone at 60 mM concentration [63,170,173]. Figure 16.4 shows an example in which the level of aggregation is reduced with increasing concentration of sucrose in an etanercept formulation at 50 mg/mL (unpublished data). However sucrose had no effect on low-molecular-weight (LMW) clips.

16.6.2. Reconstitution

The mechanism of stabilization of proteins by lyophilization has been relatively well studied, but not much is known about reconstitution of lyophilized cakes. There has been increased interest in high dose formulations of therapeutic proteins, including antibodies as an option to reduce dosing frequency. This comes with different challenges with respect to reconstitution of the lyophilized cake. Typical issues are long

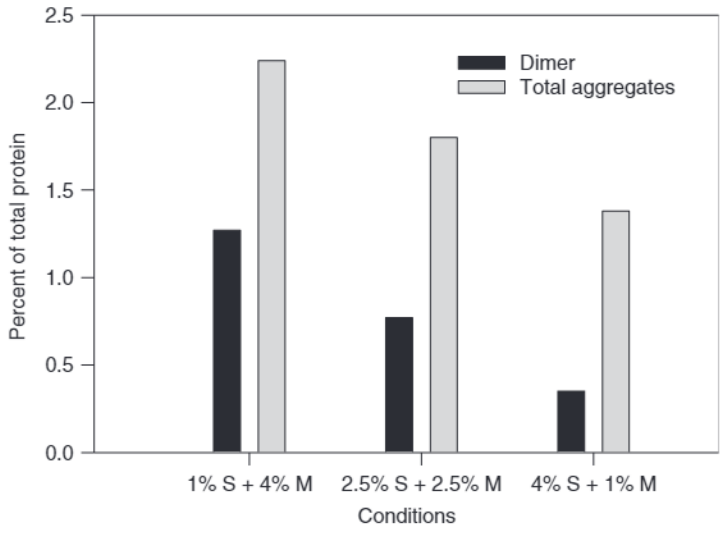

Figure 16.4. Size exclusion HPLC analysis of the Etanercept 50 mg/mL lyophilized samples on reconstitution containing different sucrose (S) and mannitol (M) concentrations. (Unpublished data.)

reconstitution times, foam layer, undissolved particles, protein denaturation, aggregation leading to turbidity, and high viscosity. The reconstitution step may potentially affect the protein stability and delivery to patients by several mechanisms. Rapid reconstitution may not allow a dried protein to rehydrate as slowly as the dehydration step [9].

We have studied the effects of different lyophilization cycle parameters, stabilizer concentration, and diluents on the reconstitution, structure, and stability of etanercept at 50 mg/ml. Annealing at −12°C and utilizing a secondary drying temperature at 25°C were important to improve the lyophilization characteristics of etanercept at high concentration (unpublished data), without compromising on the structure and stability of the protein (Table 16.3). Reducing air bubbles in the formulation prior to lyophilization and the diluent by degassing those solutions, coupled with vacuum during the freezing step of the cycle, was effective in improving the reconstitution properties of the etanercept at the high dose. Finally, diluents such as pluronic F68 decreased effervescence and foam formation during reconstitution of the cake.

16.6.3. High-concentration Strategies for Lyophilized Formulations

Noncovalent aggregation is a common limitation to high-concentration protein formulations.. To circumvent this problem, a formulation strategy was developed for proteins undergoing a slow self-association reaction. To minimize aggregation, the protein is lyophilized at low concentration in buffer containing half the standard excipient levels;

TABLE 16.3. Reconstitution Properties of Etanercept 50 mg/mL Lyophilized at Different Annealing and Secondary Drying Temperatures

Samples	Reconstitution Time, (s)	Height of Foam, mm	Comments
−20°C annealing, 45°C secondary drying	~90	6.4 ± 0.8	Effervescence observed on reconstitution; some solid material observed on the side of the vial but dissolve
−20°C annealing, 25°C secondary drying	41.6 ± 5.8	5.0 ± 1.4	Effervescence observed on reconstitution; some solid material observed initially but dissolve
−15°C annealing, 25°C secondary drying	42.5 ± 4.5	4.8 ± 1.4	Effervescence observed on reconstitution
−10°C annealing, 25°C secondary drying	68.3 ± 7.6	3.4 ± 2.2	Reduced effervescence on reconstitution compared to the other two annealing temperatures; slow dissolution of the cake
−5°C annealing, 25°C secondary drying	94.5 ± 5.0	2.9 ± 0.7	Reduced effervescence on reconstitution compared to the other two annealing temperatures; slow dissolution of the cake

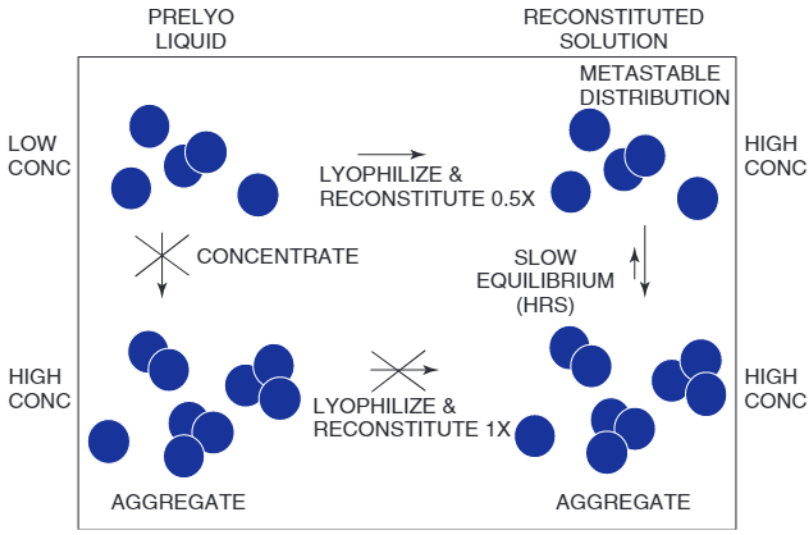

Figure 16.5. High-concentration lyophilized formulation strategy for proteins susceptible to aggregation. This strategy involves processing the protein at low concentration at 0.5× excipient levels, and then reconstituting the lyophilized cakes to half the original fill volume. (Unpublished data.)

then the lyophilized cake is reconstituted with "water for injection" to a final volume half that of the original fill volume to deliver a high concentration dosage form at full-strength excipient levels (Fig. 16.5) On reconstitution, the solution resembles the low-concentration material in terms of its aggregate content, and over the course of hours, the reconstituted solution undergoes a slow equilibrium to obtain an aggregate content characteristic of a high-concentration formulation. Assuming that the aggregation rate is sufficiently slow, the material will be injected before the aggregates grow. This strategy locks in the aggregate profile associated with the low concentration and takes advantage of the slow equilibrium between monomers and noncovalent aggregates. The method avoids processing and filling the purified bulk solution at high concentrations, where room temperature exposure may induce significant precipitation, membrane clogging, yield losses, and aggregation in the formulation. From a clinical standpoint, this strategy allows patients to have increased room temperature handling of the formulation without adverse impact on product quality attributes.

An example of the utility of the high concentration lyophilized formulation method was shown (Fig. 16.6) for r-met-Hu-Fc-Leptin (unpublished data). The protein was formulated and lyophilized at 50 mg/mL with 0.5 × excipient levels (5 mM histidine, 1% mannitol, 1% arginine HCl, 0.1% polysorbate 20, pH 5.0) and reconstituted with 0.5 × volume of water to result in a final concentration of 100 mg/mL with full-strength excipient levels (10 mM histidine, 2% mannitol, 2% arginine HCl, 0.2% polysorbate 20, pH 5.0). Aggregation kinetics were measured using size exclusion chromatography (SEC) to quantify the loss of main peak over time at room temperature

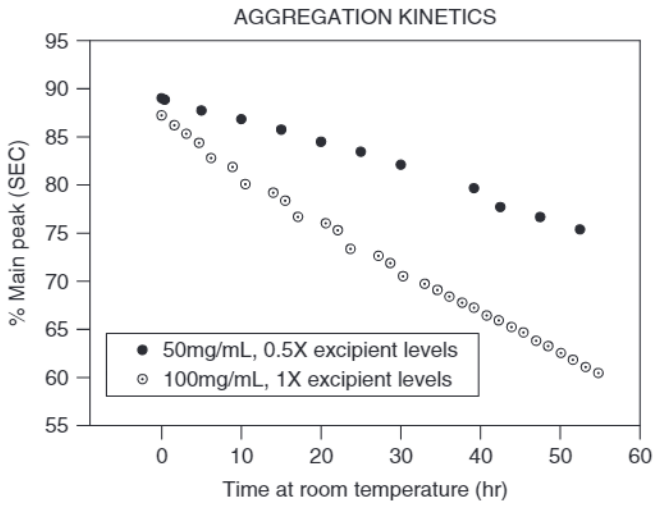

Figure 16.6. Aggregation kinetics using size exclusion chromatography to quantify the loss of main peak over time at room temperature for r-met-Hu-Fc-Leptin formulated at 50 mg/mL with 0.5 × excipient levels (5 mM histidine, 1% mannitol, 1% arginine HCl, 0.1% polysorbate 20, pH 5.0) and at 100 mg/mL with full-strength excipient levels (10 mM histidine, 2% mannitol, 2% arginine HCl, 0.2% polysorbate 20, pH 5.0). (Unpublished data.)

(Toso Haas G3000 SWxl column; mobile phase of 100 mM NaCl, 0.5 M NaCl, pH 6.9; flow rate 0.5 mL/min). The data show that the bulk drug substance at 50 mg/mL (0.5 × excipient levels) was more stable than at 100 mg/mL (1 × excipient levels), thus demonstrating the utility of this formulation strategy (Fig. 16.6) (unpublished data). This formulation strategy may be applied to other therapeutic proteins that undergo slow aggregation kinetics at high protein concentrations. Other variations on this method that employ different concentration factors other than a twofold increase in deliverable dosage may also be utilized.

16.7. ISSUES AFFECTING FORMULATION

16.7.1. Product Heterogeneity

Physical and covalent heterogeneity can confound the detection and interpretation of antibody stability properties. Known types of heterogeneity found in antibodies include glycosylation [127,128,174], *N*-terminal pyroglutamate formation [88,129], *C*-terminal processing [124–126], charge heterogeneity [78,79], disulfide connectivity [175], and conformational heterogeneity [88,122]. Reversed-phase HPLC analysis of intact antibodies revealed that the IgG2 subclass displayed significant heterogeneity, not seen in the IgG1 subclass [88,122]. Nonreduced peptide mapping and mass spectral characterization identified disulfide connectivity as the source of heterogeneity for

the IgG2 subclass [176]. Disulfide connectivity has been shown to affect the overall conformation and flexibility of the antibody [123]. The heterogeneity in the IgG2 subclass was exhibited not only for recombinant monoclonal antibodies but also in endogenous human samples. Therefore, the existence of the human IgG2 subclass as an ensemble of structural isoforms is a natural phenomenon with structural and functional implications.

Redox (reduction–oxidation) treatment of IgG2 antibodies was implemented to populate isoforms with different disulfide connectivity of the light to heavy chains [123]. Redox treatment in the absence and presence of denaturant (approximately 1 M guanidine HCl) populated the two main structural isoforms (Fig. 16.7). As confirmed by non-reduced peptide mapping, redox treatment in the presence of denaturant populates the isoform termed "IgG2-A," which contains the expected disulfide connectivity with the light chain linked to the CH1 loop of the heavy chain. In contrast, redox treatment in the absence of denaturant populates "IgG2-B," a disulfide isoform Fab arm linked to the hinge region [123]. The IgG1 subtype and each of the IgG2 isoforms have distinctly different stability properties, presumably due to the conformational and/or flexibility effects of disulfide connectivity and hinge length differences.

In addition to disulfide heterogeneity of the IgG2 subclass, redox treatment was utilized to remove cysteinylation. An IgG1 antibody was refolded to remove cysteinylation in the variable region, and the refolded form with a free sulfhydryl was more stable than the cysteinylated form by DSC, equilibrium denaturation, and stability studies [177].

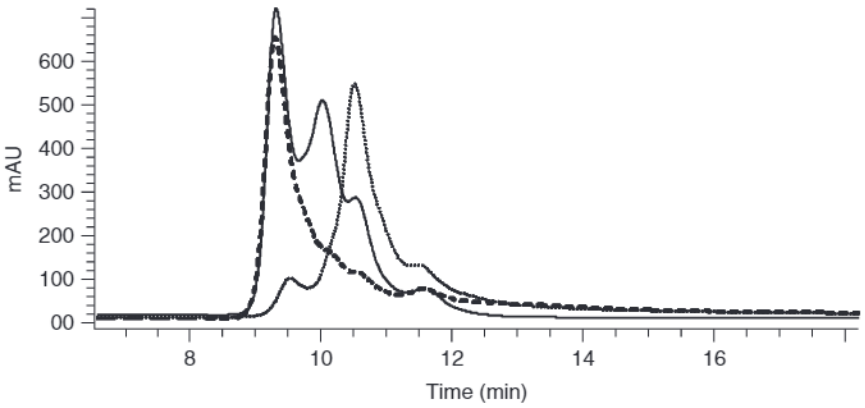

Figure 16.7. Reversed-phase chromatograms of the IgG2 control (solid line), native refold (dashed line), and guanidine HCl refold (dotted line) samples. The control represents a heterogeneous mixture of disulfide isoforms. Oxidative refolding in the absence and presence of denaturant can populate different isoforms (IgG2-B and IgG2-A, respectively). [More details can be found in Wypych et al. [176] and Dillon et al. [123].

16.7.2. Formulation Process: Filterability, Filling, and Other Operations

Antibody formulations may be impacted by processing conditions. For example, one must consider the impact of the formulation to contact surfaces such as stainless steel, nonideality effects during ultrafiltration/diafiltration operations [178], and the filling equipment. Contact with stainless steel can lead to methionine oxidation in high salt formulations [80] or in histidine solutions. Storage of high salt solutions in stainless-steel vessels can lead to pitting of the vessel and possibly leaching of metals.

As antibodies are formulated at higher concentrations, nonideality in the formulation process becomes more frequent. Excipients may coconcentrate or be excluded during ultrafiltration/diafiltration (UF/DF) operations as described by Stoner et al. [178]. At high protein concentrations, differences can sometimes be explained by use of molal concentrations instead of molar concentrations to account for volume exclusion effects. Antibodies are typically formulated away from their isoelectric point, and are charged. The Donnan effect accounts for partitioning of charged solutes across a membrane and is more pronounced in formulations with low concentrations of charged excipients. Non-specific interactions between proteins and solutes may also account for solute concentration differences across a membrane. If a solute has a positive affinity for the protein, the solute concentration in the formulated solution will be higher than diafiltration buffer. However the opposite effect may also be observed where the solute concentration is reduced by preferential exclusion [178]. These phenomena should be evaluated during formulation development, especially if much of the formulation optimization and testing are performed at lower antibody concentrations, where the effects may not be as pronounced or if multiple concentrations are desirable for the final antibody formulation.

The presence of certain formulation excipients during UF/DF can reduce turbidity or filter clogging. However, there may be other factors that preclude use of certain excipients during UF/DF. For example, during clinical development of an antibody in one study, an expensive excipient was removed from the UF/DF buffer, since 90% of the excipient went to waste when 10 diavolumes were used [179]. Experiments showed that this excipient reduced turbidity of in-process samples, but had no impact on the final product quality or long-term stability [179], justifying removal of the excipient from the process. The presence of surfactants such as polysorbates during UF/DF operations may minimize aggregation and filter clogging, but can present a control problem to consistently reach the target surfactant concentration.

The fill process itself can also lead to aggregation. Unlike the UF/DF operation, the fill process entails no further filtration; therefore, minimizing aggregation during filling is critical. Changes in fill equipment sometimes impact aggregation. For example, an antibody was initially filled using a rolling diaphragm pump, a change to a rotary piston pump in which the antibody solution, itself, is used to lubricate the piston was determined to be unacceptable [179]. Evaluation of different filler types during formulation development would allow the most flexibility; however, predicting all desired process changes is seldom feasible and needs to be dealt with on a case-by-case basis.

16.8. OVERVIEW

16.8.1. Holistic Cross-Functional Approach to Antibody Formulation Stability

Antibody formulation stability trends need to be examined holistically and appropriately balanced. Multiple mechanisms of degradation with different pH and temperature dependences can lead to complex stability trends and counterintuitive conclusions. Accelerated stability at elevated temperatures is a critical tool for formulation development [180]. An inherent assumption in the utility of accelerated data is that the stability properties at high temperatures are predictive of long-term shelf life from 2°C to 8°C. Presumably in the absence of conformational perturbations, these accelerated conditions would promote degradation reactions representative of actual storage temperatures, and the degradation reactions would follow Arrhenius kinetics [9]. However, multiple mechanisms of degradation can make the overall formulation trends complex and lead to non-Arrhenius kinetics. Degradation reactions, such as clipping and aggregation, have different temperature and pH dependences. The degradation reactions that predominate at elevated temperatures may not be the rate-limiting degradation reaction under refrigerated storage conditions. In addition, degradation reactions can be interdependent, such as the clip-mediated aggregation reaction observed in high-concentration antibody formulations at elevated temperatures.

The complex pH dependence of aggregation as a function of temperature renders formulation decisions based on accelerated data questionable. The most stable formulation at elevated temperatures may not be the optimal formulation under normal storage conditions. In addition, dramatically different aggregation properties at elevated temperatures have storage and handling implications for antibodies. This is particularly a concern for take-home products that may experience temperature excursions in transport, storage, and administration of the product by the patient. In addition, varying levels of structural isoforms between lots can result in differences in stability with respect to higher-order aggregates, clipping, temperature dependence, and so on. Differences in steric hindrance or flexibility of the hinge region may be responsible for the aggregation and clipping propensity.

Throughout the commercialization of a therapeutic, multiple processes may be used to produce the drug substance. Process changes provide benefits such as increased process yield, removal of less desirable raw materials, and reduced manufacturing time. Individual changes are typically evaluated for impact on the quality of the bulk drug substance. Evaluation of the process for impact to the formulation stability profile of the drug substance and drug product is frequently assumed to be out of scope. However, seemingly innocuous process changes can have significant impact on formulation stability for products at any stage of development. The commercial formulation recommendation for antibody therapeutics in development is based on the stability properties of early process material, but commercial process material can display unexpected increases in aggregation, particle propensity, or other degradation. These issues may manifest in the product quality of the drug substance, on freeze–thaw, or within the drug product formulation. Process changes in cell culture, viral inactivation pH, diafiltration buffer conditions, filter changes, or residual process impurities can

contribute to stability differences. The physicochemical mechanism for these stability differences can be due to glycosylation, irreversible conformational perturbation, binding effects, or product heterogeneity. Therefore, it is important to assess the impact on drug product formulation stability as part of the comparability assessment. Process and formulation scientists must communicate regarding process changes throughout the stages of commercial process development, recognize potential formulation consequences of process changes, and demonstrate that there is no impact on formulation stability.

Another challenge to antibody formulation strategy is the analytical methods available for physical and covalent characterization. In some cases, assessment of the formulation stability is limited by the quality of the analytical methods available. With more recent analytical advancements, formulation scientists are becoming aware of new and interesting chemistries and conformational heterogeneity of antibodies. In many ways, antibody stability is not as straightforward as originally assumed, and may become more challenging to demonstrate as advanced tools used to characterize heterogeneity become available.

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