Mini-Review

Theme: Aggregation and Interactions of Therapeutic Proteins

Guest Editor: Craig Svensson

High-Throughput Biophysical Analysis of Protein Therapeutics to Examine Interrelationships Between Aggregate Formation and Conformational Stability

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Abstract. Stabilization and formulation of therapeutic proteins against physical instability, both structural alterations and aggregation, is particularly challenging not only due to each protein's unique physicochemical characteristics but also their susceptibility to the surrounding milieu (pH, ionic strength, excipients, *etc.*) as well as various environmental stresses (temperature, agitation, lyophilization, *etc.*). The use of high-throughput techniques can significantly aid in the evaluation of stabilizing solution conditions by permitting a more rapid evaluation of a large matrix of possible combinations. In this mini-review, we discuss both key physical degradation pathways observed for protein-based drugs and the utility of various high-throughput biophysical techniques to aid in protein formulation development to minimize their occurrence. We then focus on four illustrative case studies with therapeutic protein candidates of varying sizes, shapes and physicochemical properties to explore different analytical challenges in monitoring protein physical instability. These include an IgG2 monoclonal antibody, an albumin-fusion protein, a recombinant pentameric plasma glycoprotein, and an antibody fragment (Fab). Future challenges and opportunities to improve and apply high-throughput approaches to protein formulation development are also discussed.

KEY WORDS: aggregation; biophysical; conformation; formulation; mini-review; monoclonal antibody; protein; stability; structure.

INTRODUCTION

Protein therapeutics are an increasingly important class of pharmaceuticals which have several advantages over small molecule drugs. These include greater specificity towards targets as well as lower toxicity. Protein therapeutics are used for a wide variety of medical applications including hormonal therapy, oncology, autoimmune disorders and as anti-infective agents. There are currently ~130 protein and peptide-based drugs approved by the FDA and there are hundreds more in the clinical development pipeline (1). As of March 2012, there were 34 monoclonal antibodies (mAbs) approved for commercial use in either the USA or Europe (2), a dramatic increase from the first approval of a mAb drug in the mid-1980s. One challenging aspect in the pharmaceutical development of protein-based therapeutics is the labile nature of their higher-order structural integrity (i.e., secondary, tertiary

and quaternary structures), which in turn, makes this class of drugs highly susceptible to degradation during manufacturing, storage and administration. When subjected to various environmental stresses (i.e., temperature, storage time, agitation, freeze-thaw, lyophilization, *etc.*), protein-based drugs can undergo chemical degradation of their amino acid residues such as Asn deamidation (3), Met oxidation (4), and polypeptide bond hydrolysis as well as physical degradation including partial unfolding, aggregation, adsorption, and precipitation (5). These chemical and physical structural alterations of a protein drug may result in sub-optimal therapeutic activity, or upon administration, can potentially generate immune responses that limit efficacy or alter their safety profile (6).

In this mini-review, we will focus our attention on one aspect of protein degradation profiles, i.e., physical instability including the interrelationships between protein aggregation and protein conformational stability. Protein aggregation is the irreversible assembly of protein molecules to form higher-order oligomers with native or non-native protein structures that may be soluble or insoluble in nature. We limit our discussion to formation of irreversible aggregates formed in pharmaceutical formulations of protein-based drugs, which tend to be amorphous in nature and typically contain intermolecular beta sheets. This phenomenon may or may not be related to the aggregation of proteins seen in a variety of human "amyloid" diseases (7). Quantitative assessment of

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protein aggregation is of particular interest to protein formulation development since the presence of protein aggregates can potentially comprise the efficacy and safety of protein therapeutics (8). Many factors affect the rate and extent of protein aggregation during storage of protein therapeutics (9) including (i) the stability and composition of the protein molecule itself (e.g., conformational stability profile and/or presence of aggregation hot spots within individual protein molecules (10)), (ii) the environment around the protein (e.g., effect of solution conditions such as pH, ionic strength and excipients) and (iii) environmental stresses (e.g., temperature, time, agitation, *etc.*). These factors in turn may not only affect the structural integrity and conformational stability of a protein molecule, but also the ability of multiple protein molecules to interact in solution to form an oligomer (i.e., colloidal stability) as well as the formation of nucleating species which in turn can promote rapid formation of aggregates in solution.

From a pharmaceutical standpoint, not only are the mechanisms of aggregate formation not well understood, but the kinetics of aggregate formation during processing and storage cannot be easily predicted, although much progress has been made in recent years (11). Additionally, due to the varied nature and composition of protein aggregates, with particle sizes ranging from nanometers to hundreds of microns (12), multiple complementary analytical techniques are required to monitor and quantitate the physical instability of protein therapeutics (13,14). Thus, minimizing protein aggregate formation is a key goal of formulation activities and requires an experimental plan to address a complex set of variables including the protein itself, formulation excipients, and solution conditions. Furthermore, the different environmental stresses on the protein solution in various primary containers (vials, syringes, stoppers, head space, *etc.*) must be considered. The utility of high-throughput biophysical analyses to monitor both protein conformational stability and protein aggregation under a wide variety of experimental conditions is therefore of growing importance. In this mini-review, we use a series of case studies to explore the ability of high-throughput biophysical analytical techniques to monitor both conformational stability and aggregate formation as part of the preformulation characterization of protein therapeutic candidates.

OVERVIEW OF PROTEIN AGGREGATION MECHANISMS

Protein Conformational Stability and Aggregation

Most proteins exist in a three dimensional folded conformation (native state) which is required for their biological activity (the exception of natively unfolded proteins has yet to prove of interest to the pharmaceutical sciences). The folded, biologically active form of a protein is a delicate structure that is only marginally stable compared to the unfolded, inactive state. There is often an experimental correlation between environmental factors and solution conditions which induce alterations of the native structure of a protein (e.g., temperature, pH, and denaturants) and subsequently observed protein aggregation. Protein aggregation may initiate without structural alterations, but often

small to large conformational changes appear to be involved. The most common intermediate structure is that of the molten globule (MG). In these states, the secondary structure remains intact, but some aspect of the tertiary structure is disrupted. In general, in contrast to older views, extensively unfolded molecules do not necessarily aggregate while MG states tend to be highly aggregative. Thus, the intrinsic conformational stability of a protein may be an important factor in its tendency to aggregation. The difference in the free energy between the folded and unfolded states (ΔG_{unf}) formally defines the extent of conformational stability, but as discussed below, other factors may contribute to protein aggregation as well.

From a pharmaceutical storage stability perspective, protein aggregation pathways (from native state to soluble to large insoluble aggregates) have been shown experimentally to proceed by a multi-stage process (many steps of which may occur in parallel). Thus, accelerated stability data following protein aggregation often cannot be described by simple two or three states models. In a series of papers (11,15), Roberts *et al.* have described a complex kinetic process, the Lumry–Eyring nucleated polymerization (LENP) model. This includes several different stages involved in the formation of aggregates as summarized in Fig. 1. The major steps include: (1) structural perturbations of the native protein, (2) reversible self-association of altered species, (3) conformational alterations which render the reversible protein–protein associations irreversible, (4) aggregate growth via monomer addition, (5) increases in aggregate size by aggregate–aggregate interactions to form larger soluble aggregates, and (6) phase separation to form insoluble aggregates. Some of the branches in the aggregation pathways (Fig. 1) involve the formation of a nucleating species, which may be as small as a dimer. The kinetics of these nucleation events do not necessarily include a lag phase. These nucleating intermediates can also potentially consist of self-associations of native monomers, structurally altered monomers or chemically modified protein. As one example, chemical changes such as methionine oxidation, deamidation, or proteolysis can alter electrostatic repulsion, alter structure or perturb the inherent solubility (16). Liu *et al.* observed that methionine oxidation in a human IgG1 Fc caused alterations in the overall secondary and tertiary structure, reduced conformational stability, and increased the propensity of the protein to aggregate (17).

Increased temperature usually induces protein unfolding which may be correlated with aggregation (18). Higher temperatures disrupt the secondary, tertiary and quaternary structure of proteins. This destabilization results in the exposure of apolar patches, often within the interior of the folded protein. These tend to act as “hot spots” in terms of initiation of protein aggregation to minimize the unfavorable exposure of these hydrophobic patches to the aqueous environment. The T_m (thermal melting temperature) of a protein is a measure of its conformational stability, and it has been observed that the T_m values of a protein under various conditions can often connect with observed aggregation rates. For example, as discussed in one of the case studies, when an IgG2 mAb was formulated with excipients resulting in higher T_m values (greater conformational stability), a lower rate of heat-induced aggregation was observed (19). For multi-

Fig. 1. Overview of various multi-step pathways observed for irreversible protein aggregation of protein therapeutics during processing and storage. Reproduced with permission from Elsevier BV (15)

domain proteins such as antibodies, structural alterations in a single domain can be sufficient to initiate aggregation of the entire molecule (20). Moreover, modifications to certain regions of a mAb, for example clipping at the hinge region of an IgG2, can create subspecies with differing conformational stability and/or tendencies to aggregate (21).

Similarly, variations in solution pH can result in changes in the charge on the basic and acidic groups of amino acid side chains in a protein causing changes in electrostatic interactions that can destabilize a protein's native structure. It has been observed that relatively small changes in solution pH can result in fairly dramatic decreases in protein conformational stability. For example, studies performed on antibodies have shown that their physical stability is highly dependent on solution pH. At pH 2, antibodies assume a structurally altered, molten-globule like state, while at pH 3.5, the Fc domain shows a loss of structure while the Fab domain remains largely intact (22). Under conditions observed during mAb purification (e.g., elution from protein A columns and viral inactivation steps), short-term exposure to low pH has limited impact on the structural integrity of these proteins, although the reversibility of these observations can vary with time, temperature, and acidic pH values (23). The correlation between solution conditions, loss of structural integrity (caused by temperature and pH) and aggregation of both model proteins (24), as well as monoclonal antibodies has been widely studied (25,26), and is discussed in greater detail through case study examples below.

Chaotropes such as guanidine HCl and urea unfold proteins by disrupting the intra molecular interactions within the molecule (27). The precise mechanisms by which these compounds exert their destabilizing effects remain an active area of study. Kendrick *et al.* studied the aggregation of a recombinant human interferon gamma in the presence of guanidine HCl. They observed an expansion of the protein's native state prior to aggregation. Sucrose which maintained the protein in its native, more compact form produced a reduction in aggregation under the same conditions. This result demonstrated that a partial structural alteration (i.e., an expansion) in the native protein conformation was correlated with the observed aggregation of the protein in solution (28). In general, it has been found that sub-concentrations of urea and guanidine HCl can induce MG states that aggregate.

Protein Colloidal Stability and Aggregation

As shown in Fig. 1, there is a reversible equilibrium between native and partially unfolded protein species. Although formation of these structurally altered species is often an initial step in the formation of irreversible protein aggregates, the proteins molecules must then assemble to form higher-order molecular assemblies which involves protein-protein interactions. For these protein-protein molecular interactions to take place, both electrostatic and steric repulsions which may inhibit such interactions must be overcome. Concomitantly, other non-covalent interactions such as van der Waals's and dipole as well as apolar attractions promote association (29). Protein molecules in solution in which the repulsive forces are greater than the attractive forces have greater colloidal stability and a lower propensity to aggregate. The extent of these intermolecular interactions can be characterized by the osmotic second virial coefficient (B_{22}) which takes into account the summation of all protein-protein interactions due to solution non-ideality (30). If a B_{22} value is positive it indicates that the repulsive forces between proteins dominate and protein-solution interaction is favored. In this case, there is greater colloidal stability which is generally preferred in the design of stable protein formulations. In contrast, if the B_{22} value is negative, the attractive forces dominate and protein-protein interactions are favored. This implies lower colloidal stability and a greater propensity towards aggregation or self-association which is undesirable for development of stable protein formulations. This is the opposite, however, if protein crystallization is desired (31). In this mini-review, we limit our discussion to protein-protein interactions leading to irreversible aggregation. Reversible protein-protein interactions, such as those observed at high protein concentrations leading to increases in solution viscosity (32), and subsequent lowering of solution viscosity upon dilution to a lower protein concentration, are not covered.

The effective charge on the surface of the protein molecule has significant impact on its colloidal stability. This effective charge is greatly influenced by the solution conditions such as pH and ionic strength. Proteins with similar effective surface charge are expected to repel each other in solution resulting in higher colloidal stability. In contrast, if a protein possesses a similar number of positive and negative groups (e.g., at a solution pH near the pI), non-covalent

attractive forces can dominate, promoting protein–protein interactions, producing a greater tendency to aggregate. For example, monoclonal antibodies with a basic pI have been found to display higher colloidal stability at lower solution pH such as 4.5–5.5, while a dramatic increase in aggregation is observed at more basic pH values closer to the protein's isoelectric point (33). The addition of salt to a protein solution will generally shield the charges on the surface of a protein. This reduces the repulsive forces between the protein molecules which in turn increases the propensity for aggregation to occur (34). Studies under stressed conditions with model proteins and antibodies have shown that conditions of pH and NaCl concentration which favor strong electrostatic repulsion result in formation of primarily small, soluble aggregates. Using solutions with higher ionic strength or more basic pH, which should suppress electrostatic repulsion between protein molecules, results in the formation of primarily larger-sized, visible protein aggregates under the same stressed conditions (35). The type and concentration of salt, as reflected in Hofmeister effects, can also play a role in the rate and extent of protein aggregation (36,37). For example, it has been reported that anions, but not cations, can selectively accumulate at protein surfaces. Depending on the anion type, varying levels of protein aggregation are observed during agitation and temperature stress of protein solutions (38,39). It has also been recently shown that salts from different parts of the Hofmeister series (a chaotrope such as NaSCN vs. a kosmotrope such as Na₂SO₄), as well as differing excipients (arginine vs. sucrose) differentially affect the local flexibility of certain regions within the C_{H2} domain of an IgG1 mAb as measured by H/D exchange mass spectrometry (40,41). These local flexibility results within the C_{H2} domain in turn correlate well with their effect on the conformational stability and aggregation propensity of this IgG1 mAb in the presence of the same compounds.

Nucleation-Dependent Protein Aggregation

Nucleation-dependent aggregation is characterized by a lag phase in which the formation of small oligomeric species occurs prior to a rapid formation of large aggregates. This is followed a rapid formation of insoluble aggregates. This lag phase is due to a defined energy barrier prior to later assembly (42). Seeding of such solutions has been shown to greatly reduce this lag phase. Protein aggregates associated with various human diseases result in formation of well defined, cross beta sheet containing fibrils which accumulate in a nucleation-dependent manner (43). With protein drugs, similar mechanisms have been observed, in which soluble aggregates can act as nucleating species. As shown schematically in Fig. 1, when these soluble aggregates reach a critical concentration, they can induce the formation of larger aggregates. Lysozyme undergoes nucleation and non-nucleation aggregation processes at elevated temperatures, depending on solution conditions and protein concentration. The nucleating pathway involves formation of small, oligomeric species (15mers or about 50 nm oligomers). These form at a different time compared to initial conformational changes in the protein's secondary and tertiary structure (44). In a study by Chi *et al.*, it was shown that although seeding of a recombinant human platelet-activating factor acetylhydrolase

formulation with protein aggregates did not significantly increase aggregation, the seeding of the protein formulation with silica particulates led to rapid aggregation. This result shows that heterogeneous nucleation can also be an important factor leading to nucleation-dependent protein aggregation in pharmaceutical formulations (45).

Interrelationships Between Protein Structural Integrity, Colloidal Stability and Aggregate Formation

As shown in Fig. 1, the aggregation behavior of protein therapeutics is often described as a combination of conformational, colloidal, and nucleation effects. Depending on the individual protein, solution conditions (pH, ionic strength, added excipients) and the type of environmental stress, one of these three mechanisms may dominate the observed aggregation pathway. We review a few illustrative literature examples below, and then in the subsequent section, present several recent case studies from our laboratories evaluating the physical stability of a variety of different proteins using high-throughput biophysical techniques.

Bajaj *et al.* studied the aggregation behavior of two proteins, a monoclonal antibody (mAb) and ovalbumin (46). Since the mAb had a pI range of 7–9, the protein was positively charged at lower pH, and charge–charge repulsion between similarly charged species was expected to reduce aggregation under these conditions. Similarly the mAb was expected to aggregate to a greater extent at a pH near its pI due to a lack of net charge. It was observed, however, that the mAb aggregated to a greater extent at pH 4 compared to pH 5.4 and 7.4. Spectroscopic analysis showed small changes in the tertiary structure of the mAb with no significant change in the overall secondary structure indicating limited unfolding of the mAb. In addition, at lower ionic strength, the extent of aggregation is lower than at higher ionic strength. These results suggested that the loss in conformational integrity was the driving force in aggregation of this mAb. Ovalbumin was also found to aggregate readily at pH 4, which is near the pI (~4.5) of this protein. Sucrose stabilized the native structure of the protein and was found to significantly reduce aggregation. Spectroscopic studies showed a significant loss in both the secondary and tertiary structure of ovalbumin during stress which was reduced by sucrose. These results again indicated that solution conditions that affected the conformational stability of the protein had a greater effect on aggregation compared to solution conditions that perturbed protein charge.

As a second example, Chi *et al.* observed that both conformational and colloidal stability play a role in the aggregation of recombinant human granulocyte colony stimulating factor (rhGCSF) depending on pH and ionic strength (47). At low ionic strength, rhGCSF was stable at pH 3.5 but aggregated rapidly at pH 7 and 6.1. In the presence of 150 mM NaCl, the protein rapidly aggregated at pH 3.5. The addition of sucrose increased the T_m and decreased the rate of aggregation at pH 7. It was, however, also observed that the ΔG_{unf} values, which are direct measures of conformational stability, were comparable for various solution conditions irrespective of the protein's differential tendency to aggregate. At pH 3.5 the molecule is highly charged and repulsive forces dominate, which explains the absence of

aggregation. In the presence of 150 mM NaCl, the charges are effectively shielded and aggregation is observed. Although the ΔG_{unf} was comparable across the three pH conditions tested, the B_{22} value was positive at pH 3.5 and negative at pH 6.1 and 7 indicating that the colloidal stability of the molecule can play a dominant role in aggregation depending on solution conditions.

As a final example, several reports have recently appeared examining the conformational stability, colloidal interactions and aggregation propensity of various monoclonal antibodies. These studies highlight that despite a high degree of overall structural similarity, individual mAbs can vary in their physicochemical and pharmaceutical properties. This variability can often be traced to differences in the constant domain in different classes of antibodies as well as to differences in the variable domains within a class of antibodies (48). The aggregation of an IgG1 mAb was examined over a wide range of salt and pH conditions. The aggregation propensity of this mAb under accelerated temperature conditions correlated primarily with the conformational stability of the Fab region with electrostatic colloidal interactions only playing a role under certain conditions (49). In contrast, optimization of pH and salt conditions, to maximize repulsive electrostatic colloidal interactions as measured by B_{22} values, were shown to be the most successful approach to minimize aggregation during 40°C storage with four different IgG2 mAbs (50). The aggregation of three different mAbs (two IgG1s and an IgG2) was examined under acidic conditions. The rate and extent of aggregation was specific to both the mAb itself and solution conditions. The results were rationalized in terms of aggregation propensity differences in the non-native, structurally-altered intermediates which formed (51). Using high-throughput techniques, Goldberg *et al.* studied the effect of pH and excipients on four different monoclonal antibodies (mAb-A, mAb-B, mAb-C, mAb-D) (52). For mAb-B, comparing the T_m values at different pH values with its aggregation tendency, conditions that resulted in a higher T_m showed a decreased rate of aggregation. For mAb-C and mAb-D, sugars increased conformational stability (raised the T_m) while salt had a destabilizing effect (lower T_m values). Sugars also minimized the aggregation of both mAbs while salt accelerated the aggregation of mAb-D only.

In summary, the studies described above reinforce the observations that each protein drug candidate can have unique physicochemical properties and aggregation behavior. Thus, as discussed in more detail in the case studies section below, to characterize, stabilize and formulate a specific protein candidate, it is not only important to evaluate solution conditions (e.g., optimal pH, ionic strength and excipients) for effects on the protein's conformational and colloidal stability, but these evaluations should be performed for each type of environmental stress (e.g., temperature, time, agitation, freeze-thaw, *etc.*) that may be encountered during manufacturing, storage and administration.

HIGH-THROUGHPUT PHYSICAL TECHNIQUES TO MONITOR PROTEIN PHYSICAL STABILITY

Because of the complex interrelationships between conformational and colloidal stability of a given protein

therapeutic under specific solution and stress conditions, there has been a growing interest in the use of high-throughput approaches for characterization during protein formulation development. This permits evaluation of a larger set of experimental conditions for a given protein by wide variety of experimental techniques, with the goal of making the protein formulation development process more efficient and productive (52–56). An overview of high-throughput screening steps for protein formulation development is shown in Fig. 2 including (1) experimental design, (2) robotic sample preparation and placement into 96 or 384 microtiter plates, (3) high-throughput screening by multiple analytical methods for physicochemical characterization, and (4) data analysis and visualization procedures (57). In this section, we review a number of high-throughput methods (58) commonly used to develop stable formulation of proteins. The measurement principle, applicability and limitations of each analytical technique are also discussed.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is a well-established technique to examine protein thermostability in solution (59). In a DSC experiment, a sample cell (containing protein plus buffer) and a reference cell (buffer only) are heated together to raise the temperature at a constant rate, and the excess heat required in the sample cell for maintaining equal temperature in both cells (due to transition from a folded, native state of protein to unfolded forms as temperature is increased) is recorded. The midpoint temperature of the thermal transition (or thermal melting temperature, T_m) is commonly used as an indicator of thermostability. Its value can be calculated by fitting the data to various transition models (59). In addition to T_m , DSC can also provide detailed information on the thermodynamic parameters of protein unfolding, including the change in enthalpy (ΔH), entropy (ΔS), Gibb's free energy (ΔG), and heat capacity (ΔC_p) (59), although experimental conditions that result in reversible thermal transitions are required. Even if the protein thermal transitions are irreversible in nature, DSC can still be used to rank order the effect of solution conditions (pH, ionic strength) (60) and excipients (22,61) on protein stability during protein formulation development. As part of lyophilization formulation development, DSC serves as the primary technique for the determination of crystallization temperature (T_c) of the frozen solution and glass transition temperature (T_g) (62) of the dried cake. One limitation of DSC is that the protein concentration that provides optimal signals during the DSC experiment may differ from the targeted protein concentration in a pharmaceutical formulation (56), although improvements in instrumentation have recently made this less of a problem.

Recent progress in automated DSC equipment has made this technique an attractive platform for high-throughput formulation development experiments. The VP-Capillary DSC platform (MicroCal, Piscataway, NJ) is specifically designed for rapid evaluation of T_m and T_{onset} values of protein samples in a 96-well microtiter plate format with a fully integrated auto-sampling system that can measure up to ~50 samples per day. For future improvements, a microelectromechanical system (MEMS)-based DSC was

Fig. 2. Overview of high-throughput screening steps for protein formulation development including (1) experimental design by scientist, (2) robotic sample preparation and placement into 96 or 384 microtiter plates, (3) high-throughput analytical methods for physicochemical characterization, and (4) data analysis and visualization techniques. Abbreviations include: *DLS*—dynamic light scattering, *DSC*—differential scanning calorimetry, *ITC*—isothermal titration calorimetry, *DSF*—differential scanning fluorescence, *Fluor*—fluorescence spectroscopy, *CD*—circular dichroism, *UV/vis*—ultraviolet/visible absorption spectroscopy, *EPD*—empirical phase diagram, *PCA/SVD*—principal component analysis/singular value decomposition. Reproduced with permission from Elsevier BV (57)

described by Wang *et al.* This novel technique reduces the requirement for sample volume from ~300–500 to ~1–2 μl , and is claimed to have a significantly broader coverage of protein concentration than currently available DSC instruments (63).

Differential Scanning Fluorimetry

Differential scanning fluorimetry (DSF), also referred to as fluorescence thermal shift assay, is another useful high-throughput technique for the characterization of protein thermostability (64). In DSF, a polarity-sensitive fluorescent dye, which emits significantly brighter fluorescence upon exposure to more apolar environments (i.e., newly exposed protein hydrophobic surfaces/patches due to structural alterations), is employed to monitor protein thermal stability by comparing midpoints of transition temperatures (T_m) (64). Commonly used fluorescent dyes include SYPRO Orange, ANS and Nile red. There have been numerous reports concerning the application of DSF to high-throughput screening of stabilizing conditions for protein therapeutic candidates (64) including monoclonal antibodies (65,66). The T_m values for various proteins in different solutions, as obtained from DSF experiments, are well correlated with T_m values determined by differential scanning calorimetry (64,66). Although non-ionic surfactants, a commonly used

stabilizing excipient, can interfere with these measurements, the use of molecular rotor dyes (67) and careful background corrections (65) have been reported to overcome these limitations.

The most popular instrument platform for DSF is real-time polymerase chain reaction (RT-PCR) instruments. The QuantiStudio 12K Flex Real-Time PCR system (Life Technologies), for example, can measure thousands of samples per experimental setup, and even more if integrated with appropriate robotics. One drawback of current RT-PCR instruments for DSF applications is that they often provide limited options in terms of excitation wavelength, limiting the number and type of hydrophobic fluorescent dyes that can be used. Use of fluorimeters, equipped with plate-reading and temperature control capabilities, are therefore another option for DSF experiments. While current available fluorimeters usually cannot match the sample throughput of RT-PCR instruments, they have the advantage of being able to accommodate a wider range of commercially available extrinsic fluorescent dyes.

Intrinsic and Extrinsic Fluorescence Spectroscopy

Other forms fluorescence spectroscopy offer well-established methods for characterizing protein conformational integrity and stability (68). Intrinsic fluorescence

spectroscopy detects the fluorescence from the internal fluorophores of proteins, such as the aromatic amino acid residues tryptophan and tyrosine. Properties of the fluorescence of tryptophan including its intensity and wavelength of maximum emission are especially sensitive to their local environment. As a result, the emission can often be used as probe to study changes in the higher-order structure of proteins. Protein unfolding is often accompanied by a decrease in fluorescence intensity and shift of maximum emission of Trp residues to longer wavelengths (red shift). Fluorometers equipped with plate reader and temperature control capability can be employed to assess the conformational stability of protein therapeutics (69–71).

Extrinsic fluorescence spectroscopy measures the emission from extrinsic fluorescent dyes. Commonly used fluorescent dyes include ANS, bis-ANS, SYPRO Orange, and Nile red (72). These dyes are largely quenched in solution, and become significantly more fluorescent when exposed to more hydrophobic environments, including for example, exposure of a protein's interior apolar surfaces due to structural changes. Extrinsic fluorescence spectroscopy can also be used to monitor protein aggregate formation (54). Further information about the application of extrinsic fluorescence dyes in protein characterization can be found in the review by Hawe *et al.* (72). Intrinsic and extrinsic fluorescence spectroscopy are commonly employed together to characterize the conformational stability and aggregation of protein drug candidates during formulation development (73,74).

There are several advantages to employing high-throughput fluorescence spectroscopic analysis of proteins during preformulation characterization and early formulation development. First, high-throughput fluorometers are widely available and have relatively low costs. Second, it is a very sensitive technique with low sample quantity requirements. Third, a relatively broad coverage of protein concentrations, ranging from picomolar to millimolar, can be evaluated enabling this technique to probe protein stability directly in formulations without the need for prior dilution or concentration of samples. Finally, as discussed below, static light scattering measurement can often be performed simultaneously permitting collection of conformational stability and aggregation data in a single experiment.

Circular Dichroism

Circular dichroism (CD) spectroscopy measures the differential absorption of left and right circularly polarized light, and is popular tool for characterizing a protein's secondary structure content (i.e., α -helix and β -sheet) as a function of temperature and solution conditions. Far-UV CD spectra (160–250 nm) are used this purpose, while near-UV CD spectra (230–320 nm) can provide information about the local environment of aromatic amino acid side chains and disulfides, which can be then be used to monitor changes in tertiary structure. One notable limitation of the CD method relates to its incompatibility with buffers and additives possessing high UV absorption. In addition, the limited sample throughput of traditional CD instrumentation has been an obstacle for its application to high-throughput formulation development. Unlike fluorescence and light scattering measurements, CD measurements are not easily

carried out in multi-well plates due to optical considerations. Recent advances in the automation of CD instrumentation, however, significantly improve its productivity and make this method more suitable for high-throughput formulation development experiments. For example, the ChirascanTM-plus ACD Auto CD Spectrometer (Applied Photophysics) is capable of accommodating four temperature controlled 96-well plates and collecting up to 200 CD scans in 24 h, using autosampling. Furthermore, under appropriate conditions, both near and far UV spectra can be obtained in a single scan (75).

Dynamic Light Scattering

Dynamic light scattering (DLS), also known as photon correlation spectroscopy or quasi-elastic light scattering, is a popular technique for monitoring changes in protein hydrodynamic properties in solution (e.g., aggregation) as well as making absolute size measurements. DLS measures the time-dependent fluctuation in the intensity of scattered light from a solution, and through autocorrelation analysis can provide information including diffusion coefficients, hydrodynamic radii, and size distribution of particles with sizes of a few nanometers up to about 1 μ m (76). A DLS signal is very sensitive to the presence of the largest sized particle in solution. This is useful for detection of higher-order protein oligomers and aggregates, but it makes it more difficult to quantitatively determine particle size distributions across a heterogeneous mixture of molecular species in solution. A major application of DLS in high-throughput formulation development experiments is to monitor the aggregation behavior of protein therapeutics (73,77). For example, DLS has been employed to characterize the colloidal stability of monoclonal antibodies as a function of solution pH and temperature (56,78). DLS has also been applied to assess the aggregation propensity of proteins in response to the physical stresses present during the production, delivery and administration of many proteins (56,79).

The DynaPro plate reader II (Wyatt Technology) is an example of an automated high-throughput DLS instrument. Three different plate formats, with 96, 384, and 1,536 wells, are available for this instrument. A typical measurement requires as little as 4 μ l of samples and takes only 4–10 s. Rapid, economic and non-invasive features makes high-throughput DLS a useful tool for protein formulation development, especially with temperature-controlled plates to automatically examine heat-induced aggregate formation as a function of time and/or temperature. DLS is not generally compatible with the study of proteins at high concentrations. When protein concentration is above 10–15 mg/ml, photons can be scattered multiple times by the protein particles in solution before being detected, making data interpretation very difficult. Interestingly, by adding polystyrene bead particle size standards to high protein concentration solutions, the viscosity of high concentration protein solutions can be determined (80).

Static Light Scattering

Static light scattering (SLS) is a technique that measures the time-averaged intensity of scattered light. It provides

information about the size of particles suspended in solution (81) and is widely used in protein formulation development (57,58,82). Multi-angle light scattering (MALS), a technique that collects and analyzes static light scattering intensity from multiple angles, can be used to determine the absolute molecular weight (83) and radius of gyration of proteins and larger molecular weight oligomers (83). MALS detection can be coupled to size exclusion chromatography (SEC) or flow field fractionation (FFF) to separate and then characterize protein aggregates (84). SLS is an attractive analytical technique due to its speed, convenience, and high sample throughput. High-throughput SLS plate readers equipped with temperature control capacity and CCD cameras can be used as the platform for characterizing the aggregation of protein therapeutics. As discussed above, fluorescence and static light scattering measurements can be performed concomitantly with a fluorometer having an additional detector typically 180° to the emission detection. Light scattering can also be measured with the fluorescence detection by simply scanning through the entire spectral region containing both the fluorescence and light scattering. This permits conformational stability and aggregation data to be obtained in a single experiment.

Turbidity Measurements

Arising from light scattering during UV-visible spectroscopic measurements, the magnitude of the turbidity (or optical density) of a solution is proportional to both the size and quantity of protein aggregates in solution (Optical density = Absorbance + Light scattering). Turbidity is usually measured in the wavelength range of 320–400 nm because proteins typically do not have significant absorbance in this wavelength range, and the magnitude of the light scattering signal is greater as the wavelength is lowered. Due to its simplicity, the turbidity method can easily be performed in a high-throughput multiwell plate format. During accelerated stability testing, the aggregation propensity of proteins in various formulations can be evaluated by either a temperature ramp method (measuring turbidity changes as a function of increasing temperature) or a kinetic method (measuring turbidity changes as a function of time at a constant temperature (85)). The turbidity method has some limitations. First, it is incompatible with samples of high opalescence solutions such as those containing a high concentration of monoclonal antibodies (86). Also, the turbidity method is not only relatively insensitive to the formation of smaller

Fig. 3. Physical stability of IgG2 monoclonal antibody solutions containing various excipients. **a** Aggregation profiles of 1 mg/ml protein at 65°C as measured by OD_{350nm}, and conformational stability of 0.2 mg/ml protein as measured by **b** intrinsic fluorescence spectroscopy and **c** differential scanning calorimetry. Reproduced with permission from Wiley Periodicals (19)

sized protein particulates, it cannot directly provide quantitative information on the size and distribution of protein aggregates without a number of assumptions. Consequently, this method is often used as a qualitative tool for assessing aggregation (e.g., in excipient screening experiments) as part of protein formulation development.

Size Exclusion Chromatography

Size exclusion chromatography, also known as gel-filtration chromatography, is a size-based separation method commonly used to characterize the size (and molecular weight or hydrodynamic radius) of proteins, and the formation of aggregates, in various formulations (87). Due to its ease of use and high reproducibility, SEC is widely considered as the standard quality control method for the detection and quantification of protein aggregates (88). Quantitative assessment of aggregate levels in protein formulations by SEC is typically accomplished by UV detection, sometimes at multiple wavelengths, and often in combination with molecular weight characterization by multi-angle light scattering detection (13). SEC can also be employed to study reversible protein self-association (89). Automated HPLC systems, with integrated and automated sample and solvent management capabilities, provide the opportunity for use with

high-throughput formulation development experiments. For example, the Alliance high-throughput HPLC system (Waters) has a robotics-based sample manager, and features the capability to process up to 1,536 samples automatically. Depending on the column used, each SEC run can potentially be finished within a few minutes. One weakness of SEC analysis is that samples get diluted during the analysis. Consequently, the results from SEC may not reflect the original properties of the sample. In addition, proteins and aggregates can potentially interact with the resin in a SEC column, which interferes with their detection and analysis (13,23). Orthogonal techniques such as analytical ultracentrifugation (AUC) and FFF are often used to confirm the results from SEC during method development (90).

CASE STUDIES USING HIGH-THROUGHPUT METHODS TO EXAMINE PHYSICAL STABILITY OF DIFFERENT PROTEINS

In this section, we present four illustrative case studies from our laboratories employing high-throughput analytical methods to assess the physical stability of different protein therapeutic candidates. These include many different multidomain proteins including a monoclonal antibody, an albumin fusion protein, a recombinant version of a

Fig. 4. Correlations of physical stability data from stressed stability studies with IgG2 monoclonal antibody solutions containing various excipients. **a** Aggregation rates (time to reach $OD_{350nm}=0.1$) with thermal unfolding temperature (T_{m1} , T_{m2} , and T_{m3}) values as measured by DSC, and **b** Aggregation rates (time to reach $OD_{350nm}=0.1$) with T_m values as measured by intrinsic fluorescence spectroscopy. Reproduced with permission from Wiley Periodicals (19)

pentameric plasma glycoprotein, and finally an antibody fragment (antigen binding fragment or Fab). These studies highlight, from a protein preformulation characterization point of view, some unique features of different proteins in different solutions exposed to different environmental stresses.

Case Study 1—Physical Stability of an IgG2 mAb

Using various high-throughput approaches, Cheng *et al.* examined the correlation between the conformational stability of an IgG2 monoclonal antibody (mAb) to the rate of aggregation under stressed conditions in the presence of ~30 different excipients (19). Initially, various biophysical techniques were used to characterize the secondary structure (circular dichroism), tertiary structure (intrinsic and extrinsic fluorescence spectroscopy), and aggregation behavior (static light scattering) of the protein as a function of pH and temperature. The data were then incorporated into an empirical phase diagram (EPD) which was used to summarize and visualize the large sets of data (70,71). For this particular monoclonal antibody, the EPD showed four distinct phases: Phase 1 (native structure from 10 to 65°C and from pH 5 to 8), Phase 2 (at lower pH values, the protein was structurally altered but not aggregated), and Phases 3 and 4 (at

temperatures above 65°C and from pH 5 to 8, the protein was structurally altered and extensively aggregated with a notable transition region, from native to aggregated state, in which the antibody had undergone observable structural changes). The aggregation propensity of the mAb at higher temperatures was monitored by following the change in optical density with time *versus* solution pH. No change in OD₃₅₀ value was observed at pH 3, but at pH 4 and 5 a gradual increase was observed with time. At pH 6, 7, and 8, an increase in the rate of aggregation of the mAb solutions was observed, but only after a certain lag period (the longest lag period was observed at pH 6). Lower temperatures and mAb concentrations increased the lag time and reduced the rate, and in some cases, the extent of aggregation.

As shown in Fig. 3, various excipients were then screened for their ability to inhibit aggregation of mAb solutions as well as their ability to enhance the protein's conformational stability. To identify stabilizers against aggregation, the time to reach an OD₃₅₀ value=0.1 was recorded (Fig. 3; top panel). As an example in the absence of stabilizers, this value was reached in ~37 min, while in the presence of sorbitol the time taken was ~490 min. The effect of the same excipients on conformational stability was then assessed using intrinsic tryptophan fluorescence spectroscopy

Fig. 5. Physical stability of albumin fusion protein (sEphB4-HSA). DSC thermograms of **a** albumin fusion protein (sEphB4-HSA), **b** sEphB4, and **c** HSA. **d** SEC chromatograms of sEphB4-HSA under native, no stress conditions (*dashed black*) and thermal-stressed conditions (*solid red*) at 48°C for 20 h. Reproduced with permission from Wiley Periodicals (91)

and differential scanning calorimetry (Fig. 3; bottom panel). In the DSC analysis, three peaks were observed with three T_m value assigned for the thermal transitions. The first peak was assigned to the C_{H2} region, the second to the Fab, and the third transition peak to either the C_{H3} region or a combination of the C_{H2} and C_{H3} region. Polyols such as sorbitol increased the T_m by approximately 5°C, while arginine decreased the T_m of the mAb. Similar experiments were performed for ~30 excipients. The correlation between the effects of these excipients on the T_m values of mAb (obtained by both intrinsic tryptophan fluorescence and DSC) and their effect on the aggregation rate (time to reach the OD₃₅₀ value of 0.1) was then examined. As shown in Fig. 4, a clear trend was observed with a correlation coefficient of approximately 0.7. The fact that the correlations between the effect of excipients on the conformational stability and kinetic aggregation profile is modest suggests that although conformational stability of the antibody plays a major role in aggregation under these conditions, it is possible that some of the excipients effect the aggregation rate in ways not related solely to conformational stability of the mAb. For example, they may directly be inhibiting the protein–protein interactions responsible for aggregate formation.

Case Study 2—Physical Stability of a Recombinant Albumin Fusion Protein (sEphB4-HSA)

The physical stability of a recombinant albumin fusion protein (sEphB4-HSA) was evaluated by Shi *et al.* (91). The recombinant sEphB4 portion blocks the interaction of two signaling factors involved in vascular development, the tyrosine kinase receptor EphB4 and its ligand, EphrinB2. EphB4 is overexpressed in a majority of epithelial cancers (92), and recombinant sEphB4 (the extracellular domain of EphB4) acts by binding to EphrinB2 and prevents its binding to the receptor. This results in inhibiting endothelial cell adhesion and migration as well as angiogenic effects of growth factors. In tumor xenograft models, sEphB4 has exhibited tumor growth inhibition (92). Recombinant sEphB4 was conjugated to human serum albumin (at the genetic level) resulting in an albumin-fusion protein with almost identical binding affinity ($K_d=3.5$ vs. 3.2 nM), but with an improved pharmacokinetic profile in a mouse model (92).

Physical stability studies were performed to compare the conformational stability and aggregation rates of sEphB4, HSA, and sEphB4-HSA. As shown by DSC analysis in Fig. 5, the fusion protein has two major thermal transitions at 57°C and 66°C and a third broad transition above 70°C. The sEphB4 protein has a single thermal transition at 57°C while HSA had a major thermal transition at 68°C and a broad transition above 70°C. These data demonstrate that the thermal stability of the fusion protein reflects the nature of each of its components. It was observed, however, that the T_{onset} of the fusion protein was lower than that of either of the individual components. A comprehensive biophysical characterization of sEphB4 and sEphB4-HSA was also performed as a function of pH and temperature using circular dichroism, intrinsic tryptophan fluorescence, ANS fluorescence, and static light scattering. EPDs were constructed as a function of pH and temperature incorporating all of the data obtained from the different techniques. Both proteins were found to be stable below 50°C

in the pH range of 5–8, but sEphB4 alone was less stable than the albumin fusion protein in the pH range of 3–5. The rate of aggregation of the three proteins was tested under accelerated stability conditions (48°C over 20 h at pH 7) by SEC (see representative chromatograms in Fig. 5; bottom right panel) and by dynamic light scattering (DLS). Results from both techniques demonstrated a time dependent increase in higher-order species for sEphB4 alone and the albumin-fusion protein sEphB4-HSA, but not for HSA alone.

Various excipients (~30) were then screened for their ability to inhibit aggregation of sEphB4-HSA solutions as well as their ability to enhance the conformational stability of the albumin fusion protein. Using high-throughput DSC measurements, 10 compounds were identified as stabilizers including sugars and both neutral and negatively charged amino acids. A combination of 150 mM NaCl and 20% sucrose increased the thermal transition temperature of sEphB4-HSA by ~15°C. Increased aggregation rate, as measured by SEC (48°C over 20 h) was observed in the presence of NaCl, but the rate was reduced in the presence of sugars. As shown in Fig. 6, plotting of the T_m observed from DSC of the sEphB4 component of sEphB4-HSA, in presence of various excipients, *versus* the aggregation rate of sEphB4-HSA (determined by SEC) with the same excipients resulted in a modest correlation ($r^2=0.79$). In contrast, no correlation was observed between the conformational stability

Fig. 6. Correlations of physical stability data from stressed stability studies with sEphB4-HSA solutions containing various excipients. **a** Aggregation rates (by SEC) *versus* thermal melting temperatures (by DSC) of the sEphB4 component of the fusion protein; **b** aggregation rate (by SEC) *versus* the thermal melting temperatures (by DSC) of the HSA component of the fusion protein. Reproduced with permission from Wiley Periodicals (91)

of the HSA component of the sEphB4-HSA with the aggregation rate of the fusion protein. These results suggest that the conformational stability of the sEphB4 component of the albumin fusion protein is the rate limiting step in the aggregation of the fusion protein under the conditions examined.

Case Study 3—Physical Stability of a Multimeric Recombinant Human Protein Pentraxin

The third case study examines the physical stability of the recombinant human protein pentraxin (rhPTX-2) (93). Pentraxins (designated as PTX-1 and PTX-2) are naturally occurring family of proteins functioning as a part of the innate immune system (94). PTX-2 consists of five noncovalently associated glycosylated protomers which form a ring like pentameric structure maintained by two salt bridges between each of the protomers as well as by hydrophobic and van der Waals interactions (95). PTX-2 has been found to regulate specific monocytes which control cellular fibrosis suggesting that this protein could be used therapeutically as an anti-fibrotic agent (96). A recombinant form (rhPTX-2) has been developed for this purpose.

Biophysical and biochemical characterization was performed as part of preformulation characterization of the protein. Upon examination by non-reduced and reduced SDS-PAGE, a single band of ~28 kDa was observed which

corresponds to the monomer. When analyzed by sedimentation velocity analytical ultracentrifugation (SV-AUC) under denaturing conditions (6 M urea), a single peak with a sedimentation coefficient of 1.1 S was identified corresponding to the monomer. Under non-denaturing conditions in PBS buffer, however, three peaks were observed, a major peak which could be attributed to a pentamer (7.4 S), and two small peaks corresponding to a decamer (11.0 S) and a higher molecular weight aggregate species (13.9 S). SEC analysis gave similar results (three peaks corresponding to the pentamer, decamer and aggregated species) as shown in Fig. 7 (left panels).

The physical stability of the pentameric rhPTX-2 protein was examined. First, a comprehensive biophysical characterization of the rhPTX-2 was performed using circular dichroism, intrinsic tryptophan fluorescence, and extrinsic fluorescence (using ANS), and optical density measurement at 350 nm to study aggregation at different pH and temperature conditions. The data from the various techniques were incorporated into an empirical phase diagram (EPD) which facilitated the visualization of the protein's structural changes as a function of pH and temperature. The protein was found to have high thermal stability. Only at temperatures above 75–80°C, were partially unfolded and aggregated states observed. Despite the high conformational stability of the protein, soluble aggregates were found to form when the protein was stored under accelerated conditions at 65°C for several days.

Fig. 7. Representative SEC chromatograms for rhPTX-2 stressed at 65°C over time (left panels), and SEC peak areas for each of three different multimeric rhPTX-2 species over time (right panels). **a, c** Samples were prepared in 10 mM phosphate buffer and **b, d** 10 mM phosphate buffer containing 150 mM NaCl at pH 7.5. Reproduced with permission from Wiley Periodicals (93)

Fig. 8. Physical stability of antibody fragment (Fab) in solutions containing varying NaCl concentrations. **a** DSC thermograms of 1 mg/mL Fab solutions containing NaCl concentrations ranging from 0 to 2 M. Thermal unfolding temperature values (T_m) are indicated for each protein solution. **b** Aggregation profiles of 1 mg/ml Fab at 67°C as measured by OD_{350nm} at various NaCl concentrations. Reproduced with permission from Wiley Periodicals (97)

Characterization of these aggregates was performed using size exclusion chromatography in the presence and absence of 150 mM NaCl as shown in Fig. 7. Both in the presence and absence of NaCl, after 3 h at 65°C, a peak corresponding to the decamer species was observed. The decamer species reached a plateau (~3 h. with NaCl and ~20 h. without) and higher molecular weight aggregates also began to form. The amount of high molecular weight aggregate was higher in the presence of NaCl. The pentamer species decreased as the decamer and higher-order species increased over time in both buffers. Generally regarded as safe (GRAS) compounds were screened using SEC to identify stabilizers against aggregation of rhPTX-2 using the accelerated stability conditions described above. The stabilizers identified included sugars (trehalose, sucrose, mannitol,

and sorbitol), amino acids (lysine, glycine, aspartic acid, and glutamic acid) as well as citrate and ethanolamine. These results suggested that multiple types of protein-excipient interactions are responsible for the stabilization of the rhPTX-2 protein against aggregation under these conditions.

Case Study 4—Physical and Chemical Stability of an Antibody Fragment (Fab)

In the final case study of this mini-review, the effect of ionic strength on the physical and chemical stability of an immunoglobulin antigen binding fragment (Fab) was examined by Wang *et al.* (97). First, the conformational stability of the Fab was analyzed at different salt concentrations (0, 0.02, 0.2, 0.5, 1, and 2 M) using various biophysical techniques as a

function of temperature. Using differential scanning calorimetry, the overall conformational stability of the Fab at different salt concentrations was evaluated. As depicted in Fig. 8, two thermal transitions are observed for the Fab at lower NaCl concentrations. As the salt concentration was increased, a single major peak was observed by DSC. The transition temperature increased from 72°C to 80°C and the total area of the DSC thermograms also increased significantly demonstrating the conformational stability of the Fab increased with increasing salt concentrations. The thermally induced aggregation behavior of the protein under different salt concentrations was then evaluated by monitoring the change in optical density at 350 nm (Fig. 8). The onset time was observed to decrease and eventually increase with increasing salt concentration from 0 to 2 M NaCl. The extent of turbidity formation, however, decreased as the NaCl concentration was increased. These results showed that the extent of Fab aggregation decreased with increasing salt concentrations during thermal stress.

The chemical stability of the Fab was assessed using ion exchange (IE)-HPLC to monitor the isomerization of Asp residues in the Fab as a function of salt concentration. The IE-HPLC chromatogram of the Fab showed peaks which consisted of a major peak (corresponding to Fab species lacking Asp isomerization), a basic peak I (consisting of LC isomerization byproducts), and a basic peak II (consisting of both LC and HC isomerization products). The percent area of the main peak from IE-HPLC was plotted as a function of time for each NaCl concentration (Fig. 9). The decay of the main peak was fit to an exponential decay function to obtain pseudo-first-order rate constants (K_{obs}). The isomerization rate was observed to decrease as the NaCl concentration was increased (Fig. 9). These results indicate a stabilization effect of NaCl towards Asp isomerization. This work not only highlights the interrelationships of solution conditions with protein physical stability, but also their effects on the chemical stability of labile amino acid residues. Interestingly, both structural alterations in native proteins as well as formation of chemical degradation byproducts could potentially serve as nucleating species to initiate subsequent formation of protein aggregates during storage.

FUTURE DIRECTIONS

Although much progress has been made in better understanding the complexities of protein aggregation pathways during manufacturing, storage, and administration of therapeutic proteins, many challenges, and opportunities remain. Despite the successful use of high-throughput biophysical instrumentation to identify formulation conditions to minimize physical stability, many of analytical techniques required to monitor protein aggregation are not easily interfaced with these platforms. For example, new analytical approaches to detect and count protein aggregates and particles in the submicron (0.1 to 1 μm) and subvisible (1–100 μm) size range, both in high and low protein concentration formulations, will need to be included in the future (14,98). Implementation of peptide mapping and mass spectrometry methods into high-throughput formulation development platforms would permit simultaneous screening of stabilizing conditions to prevent both chemical as well as physical degradation of protein-drug candidates. New and improved data collection, analysis and visualization tools remain a need since high-throughput analysis generates large data sets that can be difficult to analyze. In this regard, in addition to the ongoing use of empirical phase diagrams as described previously (70) and discussed in the case studies, recent work with new data visualization techniques including radar plots, Chernoff face diagrams and comparative signature diagrams are all being currently explored in our laboratories (74,99).

In terms of better utility for protein formulation development, expansion of capabilities for high-throughput screening of lyophilized formulations, along with the ability to evaluate the effects of different primary containers such as siliconized prefilled syringes, are needed. For example, it would be useful if the plates used to hold samples during high-throughput screening experiments could be constructed of material to better represent actual pharmaceutical containers. As high protein concentrations become more important to pharmaceutical development, other physical instability challenges such as solubility will need to be addressed. The

Fig. 9. Chemical stability of antibody fragment (Fab) solutions containing varying NaCl concentrations. Ion exchange chromatography was utilized to monitor Asp isomerization in Fab formulated in Fab solutions at pH 5.0 and indicated NaCl concentration. **a** Loss of main peak area over time; solid lines are first-order exponential decay curve fits of experimental data points. **b** Calculated values of k_{obs} derived from first-order exponential decay curves. Reproduced with permission from Wiley Periodicals (97)

use of high-throughput screening techniques to evaluate the effect of excipients on protein solubility has been described and appears promising (53).

An ongoing challenge remains the ability to utilize accelerated stability data (temperature, agitation, *etc.*) to predict real-time storage stability (11,15). This only intensifies as our ability to generate large data sets from high throughput methodologies expands. It seems likely that such correlations can be developed over time, albeit in many cases retrospectively, for individual proteins formulated in specific solution conditions, although predicative models with more general applicability remains a long-term goal. Finally, evaluation of protein degradation pathways and corresponding stability profiles during storage continues to be an important part of overall comparability assessments (100), a regulatory requirement to ensure manufacturing process changes does not affect the overall quality (i.e., structure–function relationships) of protein drugs.

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