

MINIREVIEW

Challenges in the Development of High Protein Concentration Formulations

STEVEN J. SHIRE,¹ ZAHRA SHAHROKH,² JUN LIU¹

¹Department of Pharmaceutical R&D, Genentech, Inc., South San Francisco, California 94080

²Department of Pharmaceutical and Analytical Development, Transkaryotic Therapies, Cambridge, Massachusetts 02139

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ABSTRACT: Development of formulations for protein drugs requiring high dosing (in the order of mg/kg) may become challenging for solubility limited proteins and for the subcutaneous (SC) route with <1.5 mL allowable administration volume that requires >100 mg/mL protein concentrations. Development of high protein concentration formulations also results in several manufacturing, stability, analytical, and delivery challenges. The high concentrations achieved by small scale approaches used in pre-formulation studies would have to be confirmed with manufacturing scale processes and with representative materials because of the lability of protein conformation and the propensity to interact with surfaces and solutes which render protein solubilities that are dependent on the process of concentrating. The concentration dependent degradation route of aggregation is the greatest challenge to developing protein formulations at these higher concentrations. In addition to the potential for nonnative protein aggregation and particulate formation, reversible self-association may occur, which contributes to properties such as viscosity that complicates delivery by injection. Higher viscosity also complicates manufacturing of high protein concentrations by filtration approaches. Chromatographic and electrophoretic assays may not accurately determine the non-covalent higher molecular weight forms because of the dilutions that are usually encountered with these techniques. Hence, techniques must be used that allow for direct measurement in the formulation without substantial dilution of the protein. These challenges are summarized in this review. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 93:1390–1402, 2004

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INTRODUCTION

Over the past two decades, recombinant DNA technology has led to the commercialization of many protein therapeutics requiring successful formulations. The most conventional route of

delivery for protein drugs has been intravenous (IV) administration because of poor bioavailability by most other routes, greater control during clinical administration, and faster pharmaceutical development. For products that require frequent and chronic administration, the alternate subcutaneous (SC) route of delivery is more appealing. Particularly when coupled with pre-filled syringe and autoinjector device technology, SC delivery allows for home administration and improved compliance of administration, and may

Correspondence to: Steven J. Shire (Telephone: 650-225-2077; Fax: 650-225-7234; E-mail: steve_shire@gene.com)

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result in expanded product markets. Proteins such as monoclonal antibodies are often administered with frequent dosing regimens and at high doses (several mg/kg). Two antibodies, Rituxan[®] and Herceptin[®] that have been approved for the treatment of cancer are intravenously administered in hospitals, but several programs are underway for use of monoclonal antibodies to treat diseases that may require outpatient administration, and hence require the development of SC route of administration. Treatments with high doses, e.g., more than 1 mg/kg or 100 mg per dose, require development of formulations at concentrations exceeding 100 mg/mL because of the small volume (<1.5 mL) that can be given by the SC routes. For some proteins, that have limited solubility, achieving such high concentration formulations may require the use of solubility enhancers. Even for the IV delivery route, where large volumes can be administered, protein concentrations of tens of milligram per milliliter may be needed for high dosing regimens and this may pose solubility challenges for some proteins such as cytokines and enzymes. Development of formulations at high concentrations also poses stability, manufacturing, and delivery challenges related to the propensity of proteins to aggregate at the higher concentrations. These challenges and appropriate analytical techniques for assessing high concentration protein formulations will be discussed.

SOLUBILITY CONSIDERATIONS

A potential challenge to developing a protein formulation that enables the desired dosing is achieving the required protein concentration in solution. The desired protein concentration may be difficult to achieve for some enzymes and cytokines that have limited solubility. The principles governing protein solubility are more complicated than those for small synthetic molecules,^{1,2} and thus overcoming the protein solubility issue takes different strategies. Operationally, solubility for proteins could be described by the maximum amount of protein in the presence of co-solutes whereby the solution remains visibly clear (i.e., does not show protein precipitates, crystals, or gels), or does not sediment at 30,000g centrifugation for 30 min.³ 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.^{1,3-5} 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. Proteins are also preferentially hydrated (and stabilized as more compact conformations) by certain salts, amino acids, and sugars, leading to their altered solubility.⁵ Protein solubility could also depend on the level of purity of the protein preparation; e.g., fibrinogen solubility was found to be dependent on the initial amount of protein because of the impurities and heterogeneity of the preparation.⁶ Hence, selection of a protein formulation based on preformulation work that uses materials from early process development runs may be a misguided decision and needs to be verified with representative larger scale preparations.

Determining the highest protein concentration achievable remains an empirical exercise because the lability of protein conformation and the propensity to interact with itself, with surfaces, and with specific solutes, all lead to protein solubilities that depend on the process of concentrating. Table 1 lists the various methods for concentrating a protein solution, with their advantages and practical limitations. Early in preformulation studies where material is limited, small-scale methods are needed to achieve high protein concentrations. The most mechanically gentle and rapid methods involve osmotic pressure dependent microdialysis principles.^{7,8} One such method⁷ very rapidly achieves several orders of magnitude increase in protein concentration simultaneous with buffer exchange using only ~0.1 mg protein. Though this approach is a useful tool for rank ordering of solubility in various formulation compositions, for some cytokines, it gave 10-times higher solubilities than those reached by dialfiltration/ultrafiltration on a Centricon[®] system that may result in shear induced protein denaturation and lowered apparent solubility (Shahrokh et al., unpublished results). Another approach based on osmotically driven solute concentration involves dialysis against a hygroscopic material such as Sephadex or polyethylene glycols.² This approach requires the use of high purity materials that do not result in protein degradation, and must be done with care to prevent over drying and exposure to very large surface areas since water rapidly leaves the protein solution. In addition, as polyelectrolytes, proteins impact the distribution of diffusible ions across the dialysis membrane to maintain

Table 1. The Commonly Used Methods of Concentrating Proteins

Concentrating Method	Scale	Benefits	Limitations	Used for Solubility Determination
Osmotic driven (dialysis) methods Against solutions	Few μL to few mL	Small-scale; mechanically gentle; rapid Simultaneous buffer exchange	Concentrations achieved may not translate to manufacturing scale	Yes
Against water absorbing materials			Have to buffer exchange later	
Solvent evaporation Vacuum, N ₂ flow	Few μL to few mL	Small-scale	Slow (may lead to degradation); concentrates excipients	Yes, only in solution composition reached at end of process
Lyophilization	mL	Pharmaceutically relevant and scalable	Must protect against drying induced degradation; concentrates excipients	No
Precipitation (salt or solvent; SCF)	Few μL to L	Quick and scalable	Solution composition not pharmaceutically relevant	No
Freezing	Few mL to L	Tens of fold concentration is achieved quickly	May lead to irreversible degradation	No
Chromatographic bind and elute	Few μL to many mL	Wide range of scale	Concentrates excipients too Process may damage protein Solution composition not pharmaceutically relevant Conditions may damage protein	No
Filtration Systems Centrifugal (Centricon)	Hundreds of μL to tens of mL	Moderate scale; may include buffer exchange	Adsorption losses; shear induced degradation Findings may not translate to manufacturing scale	Yes, if not material limited
Pressure (Amicon)	Hundreds of mL to L	Manufacturing scale; may include buffer exchange	Large scale impractical for early development phases	
TFF			High viscosity or pore clogging may limit concentrations reached	

electroneutrality as dictated by the Donnan equilibrium.⁹ This could lead to a potentially large change in the ion composition within the protein solution as it concentrates, and could be damaging to the protein. The alternative small-scale methods of solvent evaporation by vacuum or nitrogen flow, and precipitation by salts or solvents,^{10–12} alter the formulation composition and may also result in protein degradation due to the time or materials used in the process. Precipitation and generation of protein powders by supercritical fluids has met some success in maintaining active or structurally native proteins, but this approach remains of limited practical use particularly for small scale studies.^{13,14} Concentration of solutes by freezing¹⁵ is another approach that could achieve a high protein concentration, but alteration in the solute composition, potential damage to the protein during freezing and thawing, and difficulty in retrieving the frozen concentrate all make this an impractical approach. Chromatographic methods that bind the protein and allow elution at lower volumes is another useful tool for concentrating proteins over a large range of scale; these include ion exchange or hydrophobic interaction chromatography, but require buffer exchange after concentration. Filtration is the most common approach for concentrating proteins, and unlike many of the methods discussed is amenable to scale-up for manufacturing. There are numerous filtration systems, each with an extent of adsorption or shear-induced denaturation/aggregation that is dependent on surface area, contact materials, and flow mechanisms. The mechanical and interfacial forces imposed on protein's structure (hence its solubility) by small-scale methods may be different than those by the large-scale processes, and hence, the results obtained by such methods would have to be verified by representative manufacturing processes. The most commonly used methods for large scale manufacturing of high protein concentrations are further discussed in "Manufacturing Considerations."

STABILITY CONSIDERATIONS

Although proteins are more complex than typical small molecule pharmaceuticals, a great deal is now known about the various chemical degradations that occur in proteins, and several reviews are available.^{16–18} Many of the chemical reactions such as deamidation, aspartate isomerization, oxidation, and peptide bond hydrolysis that occur

in proteins are hydrolytically driven requiring the presence of water, and generally the kinetics follows lower order concentration dependency. Aggregation, on the other hand, requiring bi-molecular collisions, and a high concentration dependency is expected to be the primary degradation pathway in high concentration protein formulations. The relationship of concentration to aggregate formation depends on the size of aggregates as well as the mechanism of association.^{12,17} Protein aggregation may result in covalent (e.g., disulfide-linked) or non-covalent (reversible or irreversible) association. Irreversible aggregation by non-covalent association generally occurs via hydrophobic regions exposed by thermal, mechanical, or chemical processes that alter a protein's native conformation.¹⁹ When the protein-unfolding step is rate limiting, the kinetics of protein aggregation often shows pseudo-first order behavior¹⁹ rather than the expected higher order concentration dependency. Further illustration of the importance of the aggregation mechanism on the relationship of concentration to aggregate formation is shown by the inverse concentration dependency of protein aggregation that results from interaction with air–water interfaces.²⁰

Protein aggregation may impact protein activity, pharmacokinetics and safety, e.g., due to immunogenicity.^{18,21,22} Irreversible protein aggregation is recognized as a major degradation product in protein formulations, whereas reversible protein self-association is often overlooked. Reversible protein association is less studied, partly because of poor analytical methodologies (as will be discussed under "Analytical Considerations") and partly because of the perception that aggregates will not be present after typical dilutions are made prior to administration. However, if the rate of dissociation is slow, then reversible aggregation may still impact activity, *in vivo* clearance and safety. Even under conditions where the rate of dissociation is rapid, the equilibrium at high protein concentration may be shifted toward a greater amount of aggregate due to molecular crowding effects.^{23–25} In particular, 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 non-ideal solution behavior of increasing the apparent thermodynamic association constant with increasing protein concentration may be shelf-life limiting, especially when the resulting

aggregates are irreversible. In addition, after *in vivo* administration, the protein therapeutic may be placed into a crowded macromolecular environment, which shifts the equilibrium to the associated state even after dilution.

Macromolecular crowding, i.e., excluded volume effects, also can impact protein physical properties such as viscosity which can have major impact on the ability to manufacture high protein concentration formulations (see "Manufacturing Considerations") as well as on the ability to administer the protein drug by injection (Fig. 2A). In general, the viscosity of a macromolecule in solution can be expressed as a virial expansion, where the viscosity, η , can be related to the solvent viscosity, η_0 , and concentration of the protein, c_p , in g/mL by a power series²⁶:

$$\eta = \eta_0 \left(1 + k_1 c_p + k_2 c_p^2 + k_3 c_p^3 + \dots \right) \quad (1)$$

where in equation 1, k_1 is the intrinsic viscosity, which is related to the contribution from individual solute molecules, and k_2 and higher order coefficients are related to effects from interactions of 2, 3, or more protein molecules. This equation can be rewritten in terms of the specific viscosity, $\eta_s = (\eta - \eta_0)/\eta_0$:

$$((\eta - \eta_0)/\eta_0)/c_p = \eta_s/c_p = k_1 + k_2 c_p + k_3 c_p^2 + \dots \quad (2)$$

In the case of significant protein self-association that results in formation of soluble aggregates, the higher order terms will dominate and lead to large increases in viscosity as a function of concentration.

There are two key approaches to mitigating irreversible protein aggregation for improving the shelf life of high concentration protein formulations. One approach uses conditions that stabilize the native conformation of the protein, and the other, reduces molecular collisions that lead to aggregation. For the first approach, molar concentrations of osmolytes, such as sugars, are added that are preferentially excluded from the immediate environment of proteins, and result in preferential hydration.^{27,28} This preferential hydration leads to an increase in the protein's chemical potential since the increased ordering of water in the hydration shell leads to an entropic decrease for the entire thermodynamic system consisting of protein and solvent water. Since a larger surface area such as that found in unfolded proteins requires a greater number of ordered water molecules for preferential hydration, the protein will

assume a more compact folded form to resist the thermodynamically unfavorable decrease in the system's entropy. Thus, the protein will minimize the surface area available for hydration leading to stabilization of the more compact folded form, which then decreases the formation of protein aggregates. However, if the native state of the protein is capable of associating, forming aggregates of native conformers, then the addition of preferentially excluded osmolytes may actually increase the amount of protein aggregate since total surface area will be diminished following protein self association. The potential impact of reversible self-association on product shelf-life or protein physical parameters will depend on the rate of dissociation as well as the possible consequent formation of irreversible aggregates during storage at high concentration. The use of preferentially excluded osmolytes to fix the aggregation problem is limited since addition of high concentrations of excipients may also add to the viscosity and osmolality of the formulation that may render it impractical for use as a parenteral solution. Also, as already noted, the addition of these osmolytes may actually increase the amount of reversible native associated protein.

The second approach to minimize aggregation is to restrict the mobility of proteins in order to reduce the number of collisions. Lyophilization with appropriate excipients may improve protein stability against aggregation by decreasing protein mobility and by restricting conformational flexibility with the added benefit of minimizing hydrolytic reactions consequent to removal of water.²⁹ The addition of appropriate excipients, including lyoprotectants, prevents the formation of aggregates during the lyophilization process as well as during storage of the final product.³⁰⁻³² A key parameter for effective protection is the molar ratio of the lyoprotectant to the protein.³³ Generally molar ratios of 300:1 or greater are required to provide suitable stability, especially for room temperature storage. While this strategy achieves an optimal final formulation at moderate protein concentrations, if the lyophilizer is used to concentrate the protein, additional challenges can result as discussed below.

MANUFACTURING CONSIDERATIONS

Although it may be possible to create a formulation during development that has the desired solubility and stability, the design of processes

that will allow for manufacturing of the formulation at a larger scale is critical for successful implementation of bench top experimental findings. Two commonly used processes will be discussed below.

Tangential Flow Filtration (TFF)

The main technology for buffer exchange and concentrating proteins that has been used at commercial scale is TFF.^{34–36} In this technology, the protein is circulated by pumping through a series of hollow fiber tubes, which allow for the passage of water and small molecules but not large macromolecules. Attainment of high concentration formulations by TFF systems can be difficult because the required membrane flux may dictate higher concentrations at the membrane boundary than the targeted concentration. As an example, when a protein is concentrated to 100 mg/mL, the concentration at the membrane may be as high as 125 mg/mL. Depending on a protein's propensity to interact with and unfold at the surface, this could lead to decreased flux and eventual membrane clogging. Proteins are often shear sensitive, resulting in denaturation,^{37,38} and the continuous circulation through tubings and pulsation of the pumps may generate sufficient shear or cavitation to result in protein unfolding and precipitation. Although it may be possible to filter out the precipitated protein during manufacture of bulk protein, the shear sensitivity of a protein may also lead to formation of aggregates and particulates during filling of the final product that requires some sort of a pumping system. Typical filling systems include piston driven and diaphragm pumps. Piston driven pumps tend to generate more shear than rolling diaphragm pumps and this needs to be considered when filling high concentration protein solutions.

Another parameter that could limit a TFF system's ability to concentrate a protein solution is the solution's high viscosity. The viscosity increase may result in such high back-pressures during the TFF process that it may exceed the capacity of the pumps. In addition, as viscosity increases, it becomes increasingly difficult to remove the final finished product from the TFF unit, leading to economically unacceptable losses. Though these limitations may be handled by appropriate TFF equipment redesign, equipment design can be an expensive proposition. Since the viscosity of a macromolecular solution is dependent on temperature, increased temperatures

would improve the TFF process if the protein stability is not compromised. Alternatively, design of an appropriate formulation to decrease viscosity may help solve manufacturing process limitations. Hence, the formulation may need to be designed to ensure not only acceptable shelf life, but also compatibility with the manufacturing process.

Although TFF is the industry standard for concentrating macromolecules at large scale, there are alternatives. Rotating and vortex systems that enhance mass-transfer have recently been designed.^{36,39} Alternatively, any drying technique with a subsequent reconstitution at lower volumes can be used as will be discussed below.

Drying Techniques for Generating High Concentration Protein Formulations

As discussed earlier in "Solubility Considerations," a high concentration protein formulation can be achieved either by decreasing the solvent content while retaining the protein, e.g., using filtration systems, or by removal of solvent followed by reconstitution with the desired solvent volume. The latter can be achieved either by bulk processing or by dehydrating final product in vials. Bulk drying processes include spray drying,^{40,41} fluid bed drying,^{42,43} vacuum drying,^{44,45} or lyophilization in tray units.⁴⁶ Crystallization has proven to be effective in the development of insulin formulations, and shows great promise for the development of stable monoclonal antibody dosage forms.^{47,48} For parenteral protein products, bulk drying processes require operation under aseptic conditions, and have been used primarily in the aerosol industry. The alternative approach of lyophilizing sterile filtered products in vials is a cost-effective approach for generating high concentration protein formulations.

To use the lyophilizer as a concentrator, a loading volume of protein, V_L , at loading concentration C_L is lyophilized and then reconstituted with a volume of diluent, V_R , where $V_R < V_L$. The final concentration of the drug product, C_F , will then be:

$$C_F = C_L V_L / (V_R + V_S) \quad (3)$$

where V_S is the volume contributed from the remaining solids. To determine the appropriate V_R that gives a desired C_F , one can estimate V_S from the sum of the partial molar volumes of all the excipients. However, the most accurate way to achieve the target protein concentration is to reconstitute with a series of diluent volumes and empirically determine the appropriate reconstitution

volume. In addition to reaching the desired concentration, sufficient volume needs to be used in the reconstitution process to ensure that the required volume for administration can be removed from the vial. Thus, experiments may need to be designed whereby V_L and/or C_L are also varied to define the required overage in the final reconstituted product. These parameters can also have a major impact on the reconstitution properties of the final product. Figure 1 shows the effect of loading concentration on reconstitution times and morphology of the lyophilized solid. Each vial was loaded with the same total mass of protein and formulation excipients (i.e., a lower C_L required a larger V_L to maintain the loading mass). Vials were reconstituted to a final concentration of 125 mg/mL and investigated for solution clarity and disappearance of all solids after 10 min. The right side of Figure 1 shows that as the protein loading concentration is decreased, reconstitution occurs much more readily. Investigation of the morphology of the lyophilized solid by scanning electron microscopy (SEM; left side of Fig. 1) shows a very dense compact structure at 110 mg/

mL compared to a loosely packed layer of flakes at 40 mg/mL. Thus, the ease of wetting of the cake is very likely related to the differences in this morphology. SEM analysis of intermediate concentrations (not shown) demonstrates a gradual transition from a dense solid to the more loosely packed structure.

The process described above allows for designing a formulation with appropriate stability and tonicity. Although isotonicity is not necessarily required for SC administration, it may be desirable for minimizing pain upon administration.⁴⁹ Isotonicity is difficult to achieve because both the protein and the excipients are concentrated during the reconstitution process. As shown in Table 2, excipient:protein molar ratios of 500:1 will result in hypertonic preparations if the final protein concentration is targeted for >100 mg/mL. If the desire is to achieve an isotonic formulation, then a choice of lower molar ratio of excipient:protein will result in a potentially less stable formulation. This was indeed the issue involving a rhuMAb preparation where the hypertonic formulation at 500:1 excipient:protein molar ratio gave a significantly

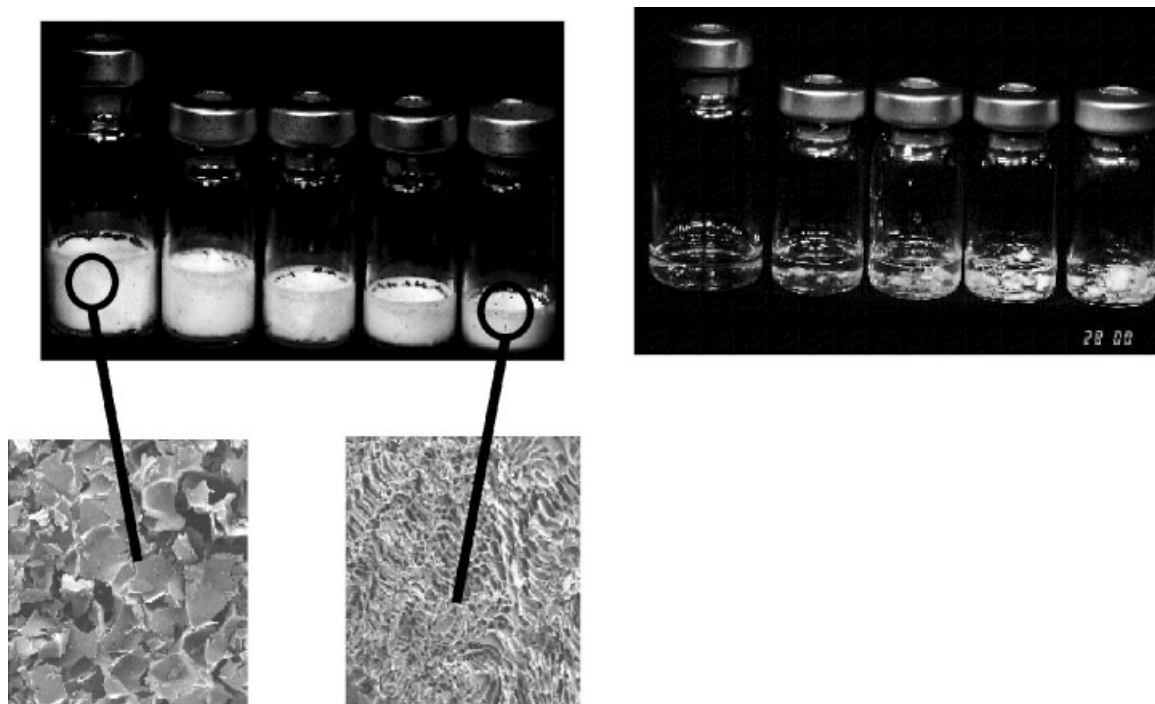


Figure 1. Lyophilization of a monoclonal antibody as a function of loading concentration. Upper left panel: Loading concentration from left to right was 40, 60, 80, 100, and 110 mg/mL, respectively, while maintaining the same total mass of MAb and excipients. Lower left panels: Scanning electron microscopy of lyophilized solid for the 40 and 110 mg/mL MAb loading concentrations. Right panel: 10 min after reconstitution of vials in upper left panel to 125 mg/mL with sterile water for injection.

Table 2. Concentration of Excipient as a Function of Final Reconstituted Protein Concentration and Molar Ratio of Excipient to Protein

Excipient:Protein Molar Ratio	250:1	500:1
C _F (mg/mL)	Excipient concentration (mM)	Excipient concentration (mM)
50	83	167
100	167	333
150	250	500
200	333	666

more stable preparation at controlled room temperature (30°C), but the stabilities at 250 and 500:1 molar ratios were comparable at 2–8°C storage (data not shown). In the case of this particular antibody where the target product profile and frequency of administration allowed for 2–8°C storage, stability was traded off for appropriate tonicity, and the 250:1 molar ratio was selected.

COST OF GOODS AND DELIVERY CONSIDERATIONS

Though high concentration formulations have the cost saving advantage of decreasing bulk storage space or number of product fills, they have undesirable overall cost of goods because of unrecoverable volumes from ultrafiltration units, fill vessels, and product containers. As an example, whereas the required volume overage in vials containing moderate protein concentrations is typically <10% of the fill volume, the overage for a >100 mg/mL protein formulation could be as high as 30% because of greater adherence of the viscous solution to product container surfaces. Unrecoverable product losses in the final dosage form could be minimized by decreasing product contact surface areas, e.g., with the use of smaller or narrower based vials, or by using prefilled syringe configurations where the solution can be pushed off the surfaces with the plunger head.

Protein formulations at high concentrations may also have physical properties that impact the ability to easily deliver the protein drug. For example, higher viscosity preparations may be difficult to administer by injection. Syringes for SC injection are often equipped with 26 or 27 gauge needles. If the viscosity of a high concentration formulation is sufficiently high, it may impact the ability to load and deliver from a syringe, and unless the viscosity can be reduced by appropriate formulation excipients, the high concentration

required for SC delivery may not be attainable. As an example, the protein concentration dependence of viscosity of an antibody is shown in Figure 2A. The time required to load 1 mL of this formulation into a syringe equipped with 27 gauge needle correlates very well with the viscosity of the formulation. Adjustment of the formulation to contain NaCl greatly reduces viscosity (Fig. 2B),

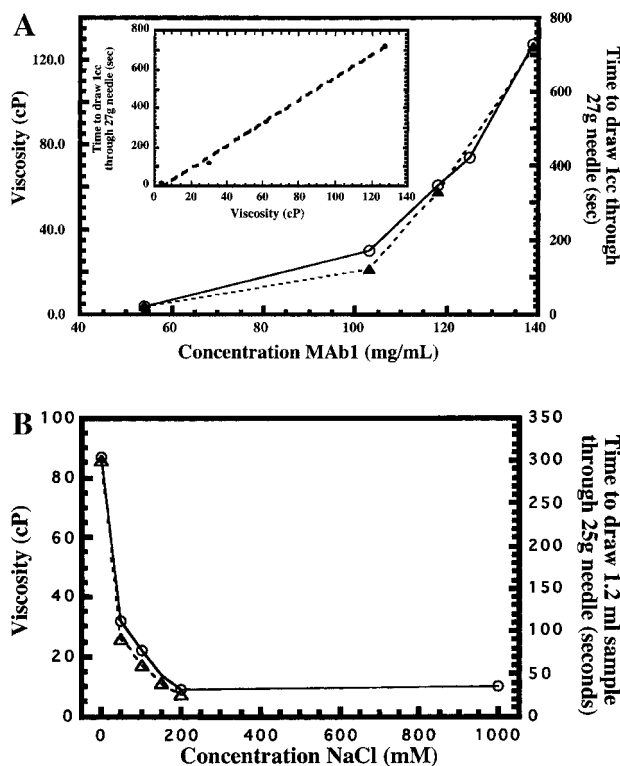


Figure 2. A: Viscosity (solid line, open circles) and syringe (27 gauge) loading time (dashed line, solid triangles) of a monoclonal antibody as a function of concentration. Insert is a linear regression fit of syringe (27 gauge) loading time versus viscosity (loading time = $-25.9 + 5.8 \times \text{viscosity}$, $R^2 = 0.995$). B: Viscosity (solid line, open circles) and syringe (25 gauge) loading time of a monoclonal antibody at 125 mg/mL as a function of NaCl concentration.

and yields reasonable times to draw the formulation into a syringe equipped with a 25 gauge needle. Thus, the alteration of the formulation excipients and the use of a slightly bigger needle yield reasonable times for withdrawal of a high concentration MAb formulation.

ANALYTICAL CONSIDERATIONS

Many of the analytical techniques currently available to explore covalent and conformational modifications in proteins^{50,51} are easily adapted to the study of proteins at high concentration. Moreover, analytical techniques that characterize solid-state protein dosage forms are useful in developing drying methods to produce a high protein concentration subsequent to reconstitution. These techniques include differential scanning calorimetry (DSC),⁵² particularly modulated DSC technology,^{53,54} to determine glass transition temperatures and thermal phase transitions of the solid-state matrix at high protein concentrations, Fourier-Transform Infrared Spectroscopy (FTIR)^{55,56} and Raman spectroscopy to study the protein's secondary structure after drying and during storage, and fluorescence spectroscopy recently used to investigate possible tertiary structural alterations during the drying process.⁵⁷

Generally the analytical technologies that are used to characterize proteins involve dilution to lower concentrations or exposure of the protein to solvent conditions that are very different than the initial formulation composition. This may have considerable impact on the results of the assay since a change in solvent composition or concentration may alter a protein's physical state in a way that is not representative of the initial conditions. This problem is especially important in the analysis of molecular weight/size distribution of proteins. SDS-PAGE, nongel sieving SDS-capillary electrophoresis, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) are often used to obtain molecular weight information. These techniques are useful for detecting covalently linked aggregates, or SDS non-dissociable aggregates but can't be used to determine non-covalent protein association states. The often-used gel permeation or size exclusion chromatographic method (SEC) provides such information but has several problems. The protein's interaction with the chromatographic resin and its hydrodynamic volume may alter the elution times; the use of globular protein

standards to estimate the molecular weight may thus be erroneous. When the interactions are ionic in nature due to the charge moieties on the resins, the addition of salts will decrease such interactions, but control experiments are then required to demonstrate that the increased ionic strength of the mobile phase did not perturb the size distribution of the protein. The impact of hydrodynamic volume leading to apparently larger proteins than actual size is especially noted for highly glycosylated proteins that have shapes different from the typical globular protein standards.^{58,59} The use of static light scattering detectors coupled with sizing chromatography (LC/LS)⁶⁰ allows for the absolute determination of molecular mass of a protein and its higher order aggregates and fragments during separation by gel sieving. Since measured elution times are no longer used to estimate molecular size, the problem of protein-resin interaction and molecular conformation are no longer important, provided the interactions do not prevent elution of the protein from the chromatographic column.

The most commonly used analytical technique for quantifying aggregates in pharmaceutical formulations is SEC with UV detection. In high concentration formulations where reversible protein self-association predominates, the determination of aggregate levels by the SEC method may be inconsistent and inaccurate. The SEC procedure involves a high dilution of the protein solution during injection onto the column that may lead to dissociation of aggregates with rapid dissociation rate constants. Moreover, to prevent detector saturation, high concentration protein solutions are generally diluted even prior to injection onto the SEC column. The rapid dissociation of a monoclonal antibody upon dilution⁶¹ yielded varying aggregate levels by SEC, depending on the time and temperature of analysis after sample dilution. To use SEC as an analytical tool for quantification of aggregates and estimation of formulation's shelf life and physical characteristics, it is imperative to have reproducible measurements. For a slower dissociating protein in the example shown in Figure 3, reproducible quantification of aggregates in an 80 mg/mL formulation is achieved by limiting the analysis time to less than 24 h and by controlling the most critical parameters, the protein concentration after dilution and sample temperature (a 5-fold decrease in dissociation rate was observed for every 10°C decrease in temperature of the auto-sampler compartment of the HPLC). The clinically relevant aggregate level for

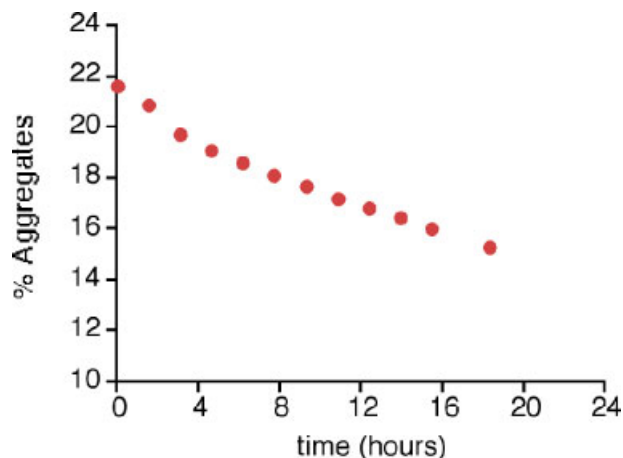


Figure 3. Dissociation of aggregates in an 80 mg/mL protein formulation at 30°C upon dilution to 0.1 mg/mL. At 5°C, the dissociation rate is too small to detect any changes in the initial aggregate level during this time frame.

a protein that undergoes reversible self-association is that which remains after dilution in the IV solution for administration (if applicable) and after initial dilution in blood volume at 37°C. This may have to be determined separately from the routine stability testing of the pharmaceutical product, particularly when dissociation rate is slower than practical for routine analyses.

Technologies such as static and dynamic light scattering^{62–65} and analytical ultracentrifugation^{62–65} provide more representative information about protein self-association states at high protein concentrations because they maintain the concentration during analysis and can also be performed at high concentration. Another technique uses preparative centrifuges coupled with a specially designed microfractionator to obtain sedimentation equilibrium measurements of molecular weight in concentrated as well as multi-component protein systems.⁶⁶ Limitations of the light scattering methods are that they are not quantitative and that they are subject to multiple scattering artifacts at high protein concentrations. Although sedimentation equilibrium centrifugal analysis can be used to characterize an aggregating system, the analysis requires fitting of data to several exponentials and is model dependent.⁶⁷ Sedimentation velocity analysis has been greatly improved and algorithms are now available to determine small amounts of protein aggregates and sedimentation coefficients.^{68,69} The determination of the sedimentation coefficient can provide valuable information regarding overall shape of

the protein when coupled with computations of hydrodynamic bead models.^{70,71} However, correction of apparent molecular weights and sedimentation coefficients due to nonideality at high protein concentrations is difficult to achieve.

SUMMARY AND CONCLUSIONS

Protein properties such as self-association/aggregation, solubility, and viscosity pose challenges to developing pharmaceutically and economically acceptable formulations at high concentration. In addition to maintaining suitable stability, protein properties at high concentration may impact the ability to administer the drug, to manufacture at large scale, and also the yields of the two processes. Analytical methodologies to investigate protein properties at high concentration are also limited and must be considered with caution as they are impacted by the very property under investigation. Very little work has been published on high concentration protein formulation development and this review has touched on the key issues with examples of the potential solutions to the issues. Achieving a suitable formulation requires an integrated approach whereby a stable formulation is developed that can also be successfully administered and economically manufactured.

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