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Review

Nature and consequences of protein–protein interactions in high protein concentration solutions

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ABSTRACT

High protein concentration solutions are becoming increasingly important in the pharmaceutical industry. The solution behavior of proteins at high concentrations can markedly differ from that predicted based on dilute solution analysis due to thermodynamic non-ideality in these solutions. The non-ideality observed in these systems is related to the protein–protein interactions (PPI). Different types of forces play a key role in determining the overall nature and extent of these PPI and their relative contributions are affected by solute and solvent properties. However, individual contributions of these forces to the solution properties of concentrated protein solutions are not fully understood. The role of PPI, driven by these intermolecular forces, in governing solution rheology and physical stability of high protein concentration solutions is discussed from the point of view of pharmaceutical product development. Investigation of protein self-association and aggregation in concentrated protein solutions is crucial for ensuring the safety and efficacy of the final product for the duration of the desired product shelf life. Understanding rheology of high concentration protein solutions is critical for addressing issues during product manufacture and administration of final formulation to the patient. To this end, analysis of solution viscoelastic character can also provide an insight into the nature of PPI affecting solution rheology.

Contents

1. Introduction

Proteins often exist in concentrated or crowded solutions. Concentrated solutions are classified as those in which a significant fraction of the solution volume, i.e. ≥0.1 is occupied by the solute molecules (Hall and Minton, 2003; Rivas and Minton, 2004). When a protein, the concentration of which might not be high in a solu-

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tion, is forced to exist in a considerably reduced volume fraction of the total solution volume due to presence of an inert solute in high concentration, the solutions are classified as crowded solutions (Minton, 2001). Concentrated and crowded solutions are often encountered in pharmaceutical milieu as in high concentration solutions of various novel proteins such as monoclonal antibodies as well as in physiological environment. The behavior of a protein molecule in such an environment is significantly affected by the presence of other like or unlike molecules. The primary consequence is the alteration of the activity or effective concentration of protein in solution, which further results in change in protein structure, function and its stability (Minton, 2005). In physiological systems, the consequences of high protein concentration coupled with rather minor structural alteration and sequence mutations are expressed in the form of various diseases and disorders due to protein assembly processes (Hardy and Gwinn-Hardy, 1998; Koo et al., 1999; Lansbury, 1999). Examples include cataract (Stradner et al., 2004), neurodegenerative diseases including Alzheimer's and Parkinson's disease (Meehan et al., 2004), systemic amyloidosis (Harper and Lansbury, 1997), polyglutamine disorders like Huntington's disease (Koo et al., 1999), etc. From a pharmaceutical perspective, high protein concentrations in solutions pose formulation challenges originating from protein solubility, manufacturing challenges due to high viscosity of some of these solutions, and often result in compromised stability of the protein in solutions with regard to self-association and aggregation (Shire et al., 2004). The term "association" or "self-association", with regard to protein solutions as used in this review and in the biophysical and pharmaceutical literature, essentially refers to the reversible formation of higher molecular weight species in which monomers in their native conformation are held together by non-covalent bonds (Minton, 2000, 2005; Attri and Minton, 2005; Schreiber, 2002). The term "aggregation", as used in this review, essentially refers to the process of formation of irreversible higher molecular weight species from the non-native monomer (Minton, 2005; Chi et al., 2003b; Krishnan et al., 2002; Roberts, 2003). The term "non-native" refers to partial or complete loss of the native structure, and confers irreversibility to the aggregates formed.

The purpose of this review is to discuss the solution behavior of proteins in concentrated solutions from a pharmaceutical perspective. The following section briefly discusses the driving force that governs the solution behavior of the proteins in concentrated solutions, i.e. thermodynamic non-ideality, which is coupled to intermolecular forces or protein–protein interactions (PPI). The next section focuses on the nature and types of these interactions that are crucial for governing the eventual micro and bulk properties of these solutions. The final section discusses the key features and reviews literature regarding physical stability and rheology of proteins in solution. These are the two more relevant consequences of PPI for pharmaceutical protein solutions in which the solute, i.e. the protein, is maintained within its solubility limit. Since our work is targeted towards pharmaceutical protein formulation development, the majority of the discussion focuses on high concentration protein solutions or concentrated solutions, which are encountered more commonly in pharmaceutical field. In these solutions, a single protein is present at high concentration and PPI between these like protein molecules is usually termed as self-interaction. A few examples of solutions exhibiting crowding induced self-association of proteins have been discussed in the later part of the text.

2. Thermodynamic non-ideality in concentrated solutions

The thermodynamic activity (a_p) or effective concentration, rather than actual concentration (c_p), of a protein in solution governs its overall reactivity and solution properties (Ross and Minton, 1977). In relatively dilute solutions when concentration of the protein is small, the activity coefficient (γ) of the protein approaches unity and its effective concentration is not significantly different from the actual concentration (Davis-Searles et al., 2001). In concentrated solutions, however, intermolecular interactions arising from the presence of greater number of solute molecules, and shorter separation distance give rise to significant deviation in activity from the actual solute concentration. A classical measure of this non-ideality is the variation in the solution osmotic pressure (Π) with protein concentration, which can be expressed as

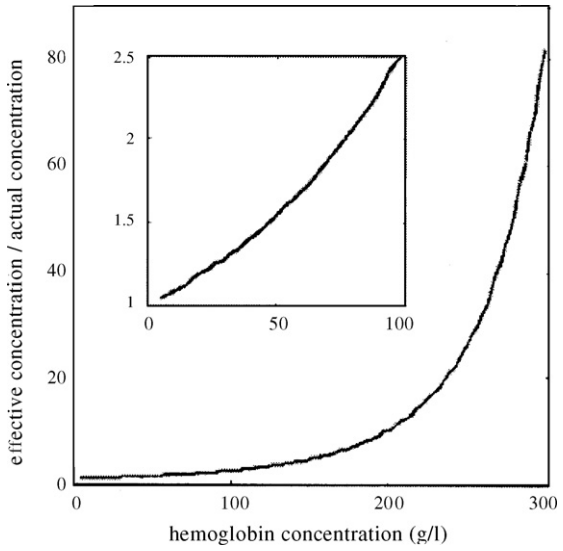


Fig. 1. Variation in the activity coefficient (effective concentration/actual concentration) with concentration for hemoglobin in solution of phosphate and potassium chloride with combined ionic strength of 300 mM (Minton, 1983).

$$\Pi = \frac{RT}{M_w}(c_p + \Gamma_2 c_p^2 + \Gamma_3 c_p^3 + \cdots + \Gamma_i c_p^i) \quad (1)$$

In the above expression, R is the universal gas constant, T the absolute temperature, M_w the weight average molecular weight of the protein molecule and Γ_i is the function expressing the potential of average force of interaction between i molecules of the protein (Ross and Minton, 1977; Zimm, 1946). The expression up to the first term represents the van't Hoff's equation for ideal solutions. The ideal solution limit is crossed as Π begins to vary non-linearly with concentration, i.e. the concentration gradient of the osmotic pressure $d\Pi/dc_p$ is no more a constant but becomes a function of protein concentration. Consequently, the measured M_w exhibits concentration dependence and is termed as an apparent M_w inversely related to $d\Pi/dc_p$. Since $d\Pi/dc_p$ is directly proportional to the variation of γ with protein concentration, i.e. $(d\ln\gamma/d\ln c_p)$, protein activity can subsequently be represented as (Ross and Minton, 1977)

$$\ln a_p = \ln c_p + B_{22}c_p + \frac{1}{2}B_{222}c_p^2 + \cdots \quad (2)$$

where B_{22} and B_{222} are the second and third virial coefficients representing the interaction between two and three protein molecules, respectively. Positive values of virial coefficients represent an increase in solute's activity over the concentration and negative values of coefficients mean decrease in activity below the actual concentration. One of the more commonly studied proteins at high concentrations is hemoglobin. A nearly 10-fold increase in the activity of hemoglobin was calculated on increasing the concentration to ≈200 g/l as shown in Fig. 1 (Minton, 2001). The activity

coefficient approached 80 as the concentration was increased to 300 g/l.

Another measure of non-ideality in protein solutions is the relative contribution from different terms in the virial expansion of the osmotic pressure, an ideal solution being the one in which the virial coefficients do not contribute to the solution osmotic pressure. Ross and Minton (1977) have conducted osmotic pressure measurements on hemoglobin solutions up to a concentration of 400 mg/ml under conditions of moderate ionic strength. Under these conditions, hydrodynamic volume of the hemoglobin molecule solely contributed to non-ideality through the effect of excluded volume, which is the volume of the solution from which the center of another solute molecule is excluded. The electrostatic contributions had been minimized by addition of salt. Even under these conditions of minimal electrostatic interactions, the authors have calculated the contributions up to the third coefficient in Eq. (2) for 100 mg/ml concentration and up to the seventh coefficient for 400 mg/ml concentration. Under the test conditions, osmotic pressure could be fitted by modeling the hemoglobin molecules as rigid spherical molecules. It could be expected that in presence of electrostatic contribution from surface charge in addition to protein volume and deviation from a spherical shape of the molecule, the relative contribution of higher order terms would further increase (Zimmerman and Minton, 1993).

The above discussion and reported results suggest that marked changes in the solution behavior of the proteins can result in concentrated solutions as compared to dilute solutions, with the effect being governed by the nature and extent of intermolecular interactions. The following section discusses the nature of these interactions and reviews some results from the literature where significant contribution from intermolecular interactions has been noted.

3. Protein–protein interactions

Protein molecules exist in solution as charged colloid particles and the potential of mean force between two or more molecules describes the phase behavior of the solution. These intermolecular interactions or PPI can be either specific, involving specific binding sites on the molecules, or non-specific in nature. The significant contributors to PPI in solution are hydrogen bonding, steric (excluded volume), electrostatic, van der Waals and hydrophobic interactions (Larson, 1999). Hydrogen bonding, van der Waals forces, and excluded volume play a relatively minor role (Norde and Haynes, 1996), whereas electrostatic and hydrophobic interactions are the major forces governing protein–protein interactions in dilute solutions of folded protein molecules (Elcock and McCammon, 2001; Curtis et al., 1998). However, the relative contribution of these forces changes with protein concentration. The strength of these PPI is expressed in terms of the potential of mean force (W), the negative derivative of which, $-\mathrm{d}W(r)/\mathrm{d}r$, measures the force between two protein molecules averaged over all possible orientations and configurations of the solute and solvent molecules (McMillan and Mayer, 1945). The symbol r represents the intermolecular center–center distance. The potential between two interacting molecules (W_{22}) can be expressed as

$$W_{22}(r) = W_{\mathrm{hs}}(r) + W_{\mathrm{charge}}(r) + W_{\mathrm{disp}}(r) + W_{\mathrm{osm}}(r) + W_{\mathrm{ass}}(r) + W_{\mathrm{dip}}(r) \quad (3)$$

In the expression, W_{hs} is the hard sphere (excluded volume) potential, W_{charge} is the energetic potential comprising of charge–charge interactions, W_{disp} is the dispersion (van der Waals) attractive potential, W_{osm} is the attractive potential due to the osmotic effect of added salt, and W_{ass} is the square-well interaction that accounts for self-association of proteins. W_{dip} represents the interactions arising from permanent and induced dipole moment of the molecules and comprises of charge–dipole, dipole–dipole, charge–induced dipole, dipole–induced dipole, and charge fluctuation contributions (Vilker et al., 1981).

The second virial coefficient (B_{22}) can subsequently be represented by the volume integral of W_{22} (Curtis et al., 1998)

$$B_{22} = -\frac{1}{2}\frac{N_{\mathrm{A}}}{M_{\mathrm{w}}^2}\int_0^\infty \left[\exp\left(\frac{-W_{22}}{kT}\right) - 1\right] 4\pi r^2\,\mathrm{d}r \quad (4)$$

The excluded volume and charge–charge contribution are repulsive in nature and result in positive virial coefficients. All other interactions listed above in Eq. (3) are attractive in nature. Of all the interactions, charge–charge interactions are significant at relatively larger intermolecular separation distance due to an inverse dependence on the first power of the separation distance. All other forces contribute significantly only when intermolecular separation distances are relatively smaller since they fall off rapidly with distance due to a higher order inverse dependence on center–center distance. Thus, in relatively dilute solutions when the intermolecular distances are large, charge–charge repulsive interactions usually contribute the most in the solution unless counter ions are present that screen these interactions. This will be discussed further in the subsequent text. The contribution of attractive forces and repulsive excluded volume effect to PPI increases rapidly with concentration due to decreasing center–center distance.

Due to large size of protein molecules, excluded volume represents a significant contribution to non-ideality in protein solutions. In order to determine the contribution of excluded volume of protein to solution non-ideality, protein molecules are usually modeled as spheres with the excluded volume approximately equal to four times the volume of the protein molecule. A contribution of hydration layer due to water molecules associated with the surface groups is taken into account in determining the hydrodynamic size of the protein molecule (Neal et al., 1998). Neal and Lenhoff (1995) have, however, shown that excluded volume contribution is significantly larger than four times the volume of the molecule modeled as a sphere. This was found to be the result of surface roughness of the protein molecules and non-uniformity in the center–center distance between protein molecules for different orientations of the molecules in solution. The authors simulated the probable configurations of two protein molecules and calculated the distance of closest approach to determine mathematically the excluded volume of the molecules. An approximate value of 6.7 times the molecular volume was determined for excluded volume. Haynes et al. (1992) have conducted osmotic pressure measurements on α-chymotrypsin in aqueous potassium sulfate solutions at 25 °C and have modeled interaction potential between protein molecules using expression similar to Eq. (3). The authors presented a truncated form of the virial expansion of osmotic pressure in which the fourth and fifth virial coefficients were based solely on excluded volume contribution. The authors also expressed the possibility of significant errors arising from assumptions regarding spherical geometry of α-chymotrypsin molecules in solution. The measurements and calculations were done up to a protein concentration of 40 mg/ml.

The charge–charge contribution to PPI is usually modeled based on Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory and assumes the counter-ions, which are usually salt ions in the case of protein solutions, to be point charges. This assumption is not valid at ionic strengths greater than 0.1 M. However, since electrostatic repulsive interactions are usually screened out at these ionic strengths, DLVO theory can be used to predict the solution behavior in lower ionic strength solutions (Curtis et al., 2002). The

electrostatic charge potential of interaction is represented by Eq. (5)

$$W_{\text{charge}}(r) = \frac{z^2 e^2 (1/r) \exp[-\kappa(r - d_p)]}{4\pi\varepsilon_0\varepsilon_r(1 + \kappa d_p/2)^2} \quad (5)$$

In Eq. (5), z is the protein valence, e the elemental charge, $4\pi\varepsilon_0$ the dielectric permittivity of free space, d_p the protein diameter modeled as a hard impenetrable sphere, and ε_r is the dielectric permittivity of water and κ is the inverse Debye length. W_{charge} is thus directly related to the protein valence and is inversely related to the solution ionic strength through the inverse dependence of Debye length on square root of ionic strength. Eq. (5) holds for conditions with $r > d_p + 2\gamma$, where γ is the hydration layer thickness of the protein. The combined effect of this dependence of W_{charge} on valence and ionic strength can be understood in terms of the effective charge of the molecule in solutions, which can be quantitated by measurement of zeta potential of the protein molecule. Zeta potential is defined as the potential at the slipping plane of the ions that move with the charged macromolecule in solution. A macroion like protein is associated with oppositely charged ions in aqueous solutions that form a rigid "stern layer" at the protein surface followed by a more diffuse layer of counter ions. Slip plane usually lies within this diffuse layer. An increase in the ionic strength leads to a decrease in the zeta potential due to presence of greater number of counter ions. Due to this inverse dependence on ionic strength, electrostatic repulsive contribution to PPI usually decreases with addition of salt ions in the solutions and this is reflected in decreasing values of virial coefficients with increasing ionic strength. Under conditions of a net charge, a number of protein molecules have been analyzed for the variation in virial coefficients with salt and have been found to exhibit this predicted trend. Examples include ovalbumin in ammonium sulfate and potassium isothiocyanate solutions (Curtis et al., 2002), lysozyme (Rosenbaum and Zukoski, 1996; Bloustine et al., 2003), ribonuclease A (Tessier et al., 2003), β-lactoglobulin (George et al., 1997), myoglobin (Tessier et al., 2002), malate dehydrogenase (Costenaro et al., 2002), etc.

However, DLVO theory cannot always explain the protein behavior in solutions of increasing salt concentration since in some instances a decrease in intermolecular charge–charge repulsions is not observed with increasing ionic strength. This is especially true for those proteins that bind salt ions, which leads to change in the effective charge or zeta potential either above or below the expected value. Apoferritin molecules in sodium acetate solutions exhibit a decrease in second virial coefficient with ionic strength up to 0.15–0.20 M followed by an increase as shown in Fig. 2 for studies conducted at pH 5.0 (Petsev et al., 2000). The p*I* of the protein is 4.0 and apoferritin molecules bear a net negative charge at the studied pH. The authors attributed the increasing repulsion, with increase in ionic strength above 0.15–0.20 M, to non-DLVO hydration, i.e. solvent-mediated forces originating due to accumulation of the hydrated counter ions (Na^+) near the protein surface (Petsev and Vekilov, 2000; Israerlachvili, 1992). The molecular origin of these hydration forces is, however, not yet clear. Different authors attribute these forces to different solvent and hydrated ion based factors. Besseling (1997) and Forsman et al. (1997) have proposed that these forces originate due to a structuring of the water layers around the interacting surfaces. The magnitude of this repulsion will depend in the nature of surface–water interaction (Forsman et al., 1997). Israelachvili and Wennerstrom (1996), however, argue against this water structure mediated effect and propose that the property of water beyond the first layer of adsorbed ions is essentially same as the bulk water. The authors propose that the repulsion occurs due to confinement experienced by the otherwise mobile surface adsorbed groups as the two surfaces approach to within small distances. Henderson and Lozada-Cassou (1994)

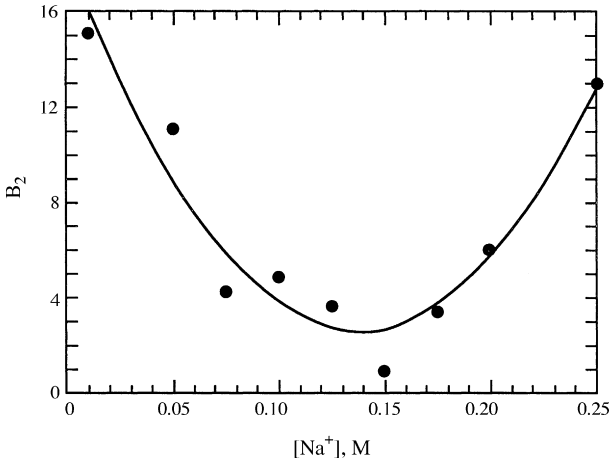


Fig. 2. Second virial coefficient for apoferritin molecules with changing sodium ion concentration at pH 5.0. The solution ionic strength was modified by changing the buffer (sodium acetate and acetic acid) concentration (Petsev et al., 2000).

and Trokhymchuk et al. (1999) propose that this hydration repulsion originates due to contribution from exclusion effect (due to a finite size of solvent molecules) and a second effect originating from orientational ordering of solvent molecules near the colloidal particles.

Divalent cationic salts like $MgCl_2$ and $CaCl_2$ offer other instances in which an increase in protein solubility and intermolecular repulsion is observed at salt concentrations between 1.0 and 3.0 M due to preferential interaction of the divalent cations with the protein molecules. Arakawa et al. (1990) have observed an increase in the preferential salt binding parameter $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ for β-lactoglobulin, bovine serum albumin (BSA) and lysozyme in aqueous $MgCl_2$ solutions. In the expression, m_2 and m_3 represent protein and salt molality and μ_1 and μ_3 represent the chemical potential of solvent and salt. Similar observations have been made for ovalbumin in $MgCl_2$ solutions (Curtis et al., 2002). At salt concentrations lower than 1.0 M, a decrease in repulsive PPI was observed with increasing ionic strength for both the proteins and no preferential binding of salt to the protein was detected. Neal et al. (1999) have measured the osmotic second virial coefficients for chymotrypsinogen and have noted a decrease as well as an increase in B_{22} values as a function of solution ionic strength depending on pH of the solution. The data is shown in Fig. 3. A decrease in B_{22} with ionic strength, consistent with the DLVO theory, was observed for

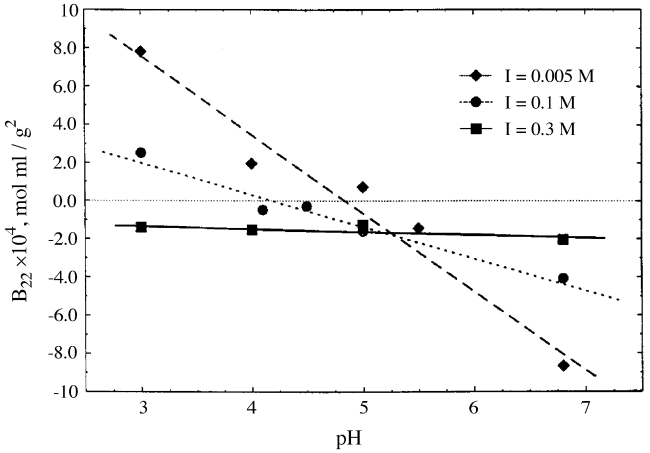


Fig. 3. Osmotic second virial coefficients for chymotrypsinogen as a function of solution pH and ionic strength (Neal et al., 1999).

pH from 3.0 to 5.0 whereas the reverse effect was observed from pH 5.0 to 7.0. As ionic strength was increased up to 0.3 M ionic strength, the effect of pH on B_{22} was neutralized.

The above discussion implies that charge–charge contribution to PPI for protein solutions cannot always be explained in terms of an idealized DLVO model based colloidal system approximation, although such an approximation is valid for a number of studied proteins (Piazza and Iacopini, 2002). This would be especially true for high concentration protein solutions where the center–center distance is not large compared to the inverse Debye length and for solutions at higher ionic strength where salt ion binding to protein molecules may be significant.

Another type of interactions that contribute to overall PPI and are not accounted for by the DLVO model for pairwise potential are the protein dipole interactions. This is especially true for solutions of low ionic strength (Tanford, 1961). Coen et al. (1995) have determined the nature of PPI in solutions of chymotrypsin and found a progressive decrease in B_{22} as pH approached p*I* (8.3) at 0.01 M sodium phosphate concentration, i.e. the interactions became attractive as p*I* was approached. The electrostatic nature of the involved interactions was confirmed by increasing the salt concentration to 1.0 M when the B_{22} increased, with the maximum effect being observed at pH 8.3. The authors have attributed the decrease in B_{22} with increase in pH at 0.01 M to charge–dipole and dipole–dipole attractive interactions.

van der Waal's forces comprising of Keesom, Debye, and London contributions are dispersive forces that are active at relatively shorter distances as compared to charge–charge interactions. These contribute significantly to overall PPI when the center–center distance is small relative to the diameter of the protein molecule. These forces depend on the nature and composition of the solute, the chemical nature of the solvent, and the geometry of the interacting species. The potential for van der Waal's forces varies with the inverse of the sixth power of the center–center distance. Thus, in going from dilute to concentrated solution regime, their contribution to overall PPI increase rather sharply. This is due to a decrease in the intermolecular center–center distance with increasing solute concentration. Roth et al. (1996) have calculated the values for the Hamaker constants for different proteins after incorporating the contribution of molecule geometry in addition to protein sequence. The authors concluded that by assuming a spherical geometry of the interacting molecules, the contribution of van der Waal's interactions to potential of mean force is often overestimated during mathematical analysis. This is because a greater number of configurations are possible in which two spherical molecules can approach each other closely as compared to irregularly shaped molecules. Irregularity in the molecular shape and surface roughness restricts the number of configurations for closest approach thereby reducing the magnitude of van der Waal's forces. The authors further discuss that if geometric complementarity, which allows the molecular sites to approach within small distances, exists between the interacting molecules, strong van der Waal's interactions can result. This kind of interaction plays significant role during molecular recognition in biological processes (Yi et al., 2007; Murthy, 2006; Iyer et al., 2005; Roth et al., 1996). Neal et al. (1999) have found a strong dependence of interaction potential on mutual orientation of interacting molecules (structural complementarity) with complementary configurations resulting in significant attractive dispersion interactions. Thus, van der Waal dispersive forces would significantly contribute to PPI in pharmaceutical solutions when the protein concentration is high enough to reduce the center–center distance to a value significantly less than the molecular diameter and a large number of complementary configurations exist that allow the distance of closest approach between interacting sites to be small.

Osmotic contribution from salt ions resulting in attraction between charged macromolecules can arise in solutions of high salt concentrations (Record et al., 1998). Under these conditions, the gradient in salt concentration in the region between the macromolecules and the bulk alters intermolecular interactions. This usually occurs when the intermolecular distance between solute molecules reduces to the order of the salt ion size, and the salt ions are squeezed out of the region between the solute molecules (Wu et al., 1998). Wu et al. (1998) have conducted simulations on the distribution of divalent and monovalent salt ions in the vicinity of two closely placed charged macromolecules and have concluded that stronger attractive effect is induced by divalent salt ions as compared to monovalent ions. Curtis et al. (1998) have also discussed this osmotic contribution to overall PPI and have indicated a sharp fall in osmotic interactions at intermolecular distances greater than the combined center–center distance and small ion diameter.

Self-association results from a direct physical contact between the interacting molecules and is reversible in nature. Proteins self-associate due to short-range interactions including hydrophobic, hydrogen, and ionic bonds. The phenomenon of self-association will be discussed in detail later in the text with discussion on physical stability and rheology of protein solutions.

All the interactions discussed above play a vital role in governing the overall solution behavior of proteins although their relative contribution is a matter of solution condition and the involved protein molecules. However, the behavior of all protein solutions, especially those at high protein and salt concentrations cannot be explained or predicted based on these interactions. This is because various assumptions valid for dilute solutions of point charges based on which an understanding of these forces has been developed, collapse or are not applicable to concentrated solutions of macroions. Vilker et al. (1981) have summarized the results of osmotic pressure measurements on high concentrations solutions of BSA and have concluded that equations relating the potentials of these forces to solute and solvent properties "represent an overly simplistic picture of BSA intermolecular interactions". The authors have outlined various deficiencies in the approach usually adopted for predicting solution properties based on these relationships and their conclusions can be summarized as follows: (i) an understanding of electrostatic charge–charge and dipole interactions is valid at large distances and is not readily applicable at small intermolecular distances, (ii) the contribution from these forces are based on assumptions of point charges, an incorrect assumption for protein molecules as well as small ions, (iii) charge distribution is not uniform throughout the macroion and (iv) the correct values of Hamaker constants for proteins are uncertain. Wu et al. (1998) and Haynes et al. (1992) have also reached similar conclusions.

4. Pharmaceutically relevant consequences of protein–protein interactions in solution

Strong intermolecular PPI in protein solutions are manifested in the form of deviations from ideal behavior of different solution properties including osmotic pressure, density, flow behavior, molecular diffusion, scattered light intensity, sedimentation, etc. From a pharmaceutical perspective, the more relevant consequences of varying PPI in solutions are changes in protein's true (thermodynamic) and apparent solubility. True or thermodynamic solubility refers to the concentration of the protein in a solution, which is in equilibrium with a crystalline solid phase of that protein (Arakawa and Timasheff, 1985). Apparent solubility refers to the concentration of protein in a solution, which is in equilibrium with a solid amorphous precipitate of the same protein. However, for solutions with protein concentration maintained below the solubil-

ity limit (true as well as apparent), self-association, and aggregation are the critical issues that challenge the physical stability and safety of protein formulations. Another issue at high protein concentrations is that of solution viscosity. It has been observed that for proteins, slight modifications in the amino acid sequence or solution environment can dramatically affect the solution viscosity (Liu et al., 2005). High viscosity of these solutions raises concerns during large-scale manufacture, processing, pumping, and even during administration of the parental products through syringes. It also imposes increased financial costs due to losses from unrecoverable product from production and processing vessels. Key features of these two aspects, i.e. physical stability of proteins in solution and protein solution rheology are discussed in the following subsections and some relevant literature is reviewed.

4.1. Physical stability

Maintaining a protein molecule in its native conformation and non-aggregated state is required to ensure the long-term safety, efficacy, as well as elegance with respect to visual appearance and clarity of a liquid protein formulation. This is a requirement not only during storage of the formulation for the duration of its shelf life but also during product processing and manufacture, handling, and delivery of the product to the patient (Chang and Hershenson, 2002). However, achieving these goals is not trivial and is often a challenge. Protein molecules are complex chemical entities consisting of a number of different chemical groups and their native structure is stabilized by a variety of different forces. The energy difference between the native and unfolded state of the protein molecule in solution is often not more than the energy of three to four hydrogen bonds (Privalov, 1979), i.e. the native conformation is only marginally stable. Consider this in addition to the fact that protein molecules in a solution are dynamic moieties, rather than being static in time, that have often been termed as "breathing" molecules (Chou, 1985; Wu and Prausnitz, 2002). Due to this diverse chemical and dynamic physical nature of protein molecules, multiple pathways exist that can result in a chemical or physical change of the native monomeric state. The chemical pathways for such a change usually comprise of deamidation, oxidation, hydrolysis, disulfide formation, racemization, etc., whereas physical challenges include self-association and aggregation. Either one of these can ultimately result in altered solubility, activity, pharmacokinetics, toxicity, or immunogenecity of the final product. Whereas chemical degradation is usually first or pseudo first order with respect to protein concentration, physical instability as measured by formation of oligomers and aggregates usually follows higher order dependence on concentration and is expected to be the more severe challenge for successful development of stable high protein concentration solution formulations (Shire et al., 2004).

4.1.1. Self-association

Self-association proceeds through the formation of dimers or oligomers from a native species and the reaction kinetics can greatly vary such that it may take a few seconds or even days and months to establish equilibrium once the solution conditions have been modified. Although self-association is reversible in nature, it can potentially affect the *in vivo* safety and storage of the formulation (Shire et al., 2004; Cromwell et al., 2006a,b). Self-association entails the existence of higher molecular weight species in solution with a consequent increase in short-range interactions. Generation of covalent linkages in these reversible associates during storage can also have a potential impact on the rate of formation of irreversible aggregates (Cromwell et al., 2006a). In these cases, the reversible oligomers could be regarded as precursors of irreversible aggregates (Philo, 2003). Further, slowly dissociating high molecular weight oligomers can have prolonged clearance half-lives and also present immunogenic challenges on subcutaneous administration (Cromwell et al., 2006b; Shire et al., 2004). As an example, Apo2L/TRAIL solutions with 25% hexamer content, when diluted to 1 mg/ml in neutral pH buffers containing 0.05 and 0.5 M NaCl, exhibited dissociation half-lives of ~90 and ~50 h, respectively (Cromwell et al., 2006a). Slower dissociation rates can also result from slow dilution of the injected preparation, which is usually the case with subcutaneously administered preparation (Cromwell et al., 2006a). In instances where reversible oligomers result in visually opalescent solutions, self-association can lead to a reduced patient confidence in the product.

An increase in protein self-association has been suggested as well as demonstrated in the presence of high concentrations of crowding agent (Wilf and Minton, 1981; Bosma et al., 1980). Self-association in these systems is induced due to the excluded volume contribution from the crowding agent and occurs in order to reduce the system free energy by decreasing the total excluded volume (Shearwin and Winzor, 1990; Minton, 2001). Wilf and Minton (1981) have studied the solution behavior of myoglobin in the presence of crowding proteins including lysozyme, ribonuclease-A and β-lactoglobulin present at a concentration of up to 250 mg/ml. The authors observed an increase in the reversible dimer formation of myoglobin with increasing concentrations of the crowding proteins. The dimer formation became evident around ≈50 mg/ml of β-lactoglobulin, ≈60 mg/ml of added lysozyme, and ≈150 mg/ml of ribonuclease A. The nature of the dimer formed was independent of the added protein suggesting that myoglobin was self-associating and not associating with the added proteins. Shearwin and Winzor (1988) have studied the solution behavior of α-chymotrypsin as a function of solution volume occupied by an inert solute by sedimentation equilibrium studies. The volume occupancy in this case was, however, provided by a small solute, sucrose. The authors have reported an increase in the extent of dimerization of α-chymotrypsin with increasing concentration of sucrose arising due to the excluded volume of sucrose. Similar results were obtained for solutions containing glycerol.

Analytical ultracentrifugation (AUC) has been traditionally used for detecting the presence of self-association in dilute protein solutions. Rivas et al. (Rivas et al., 1999a,b, 1994; Rivas and Minton Allen, 2004; Rivas and Minton, 2003) have extensively investigated the technique of tracer sedimentation equilibrium (TSE) in analyzing non-ideality, self-association, and hetero-association in crowded systems. The technique involves conducting sedimentation equilibrium experiments on a series of solutions of a tracer protein in the presence of a crowding agent. The tracer protein is present at a fixed concentration in all the solutions, whereas the concentration of the crowding agent is increased in the series of solutions. The requirement is that the tracer concentration should be measurable independent of the other solutes present in the solution. In one of the studies (Rivas et al., 1999a), solution behavior of labeled fibrinogen (tracer) was analyzed in the presence of increasing concentrations (0–100 mg/ml) of bovine serum albumin (crowding agent) in the presence and absence of Ca^{2+} and Mg^{2+} ions. In the absence of cations, a decreasing apparent molecular mass of fibrinogen with increasing BSA concentration was observed. The decrease in molecular mass was caused by a predominant contribution from thermodynamic non-ideality due to steric repulsion of fibrinogen by BSA molecules. However, in the presence of cations, the molecular mass of fibrinogen increased with increasing BSA concentration due to self-association. The molar mass of fibrinogen nearly doubled at 40 mg/ml BSA concentration. In the same work (Rivas et al., 1999a) labeled tubulin was also found to self-associate in the presence of increasing concentration of dextran.

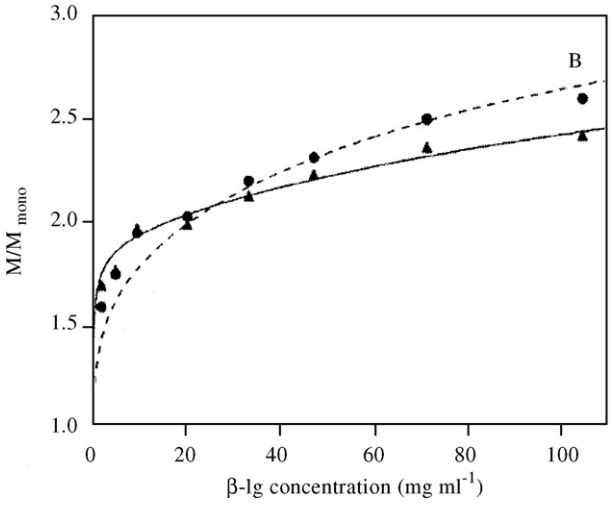


Fig. 4. Effect of protein concentration on the ratio of the weight average molecular weight to the monomer molecular weight (M/M_{mono}) for beta-lactoglobulin (Verheul et al., 1999) at pH 6.9 and 0.1 M NaCl. The symbols represent data acquired at (▲) 20 °C and (●) 45 °C. The lines represent the fit of a monomer–dimer and a dimmer–tetramer model to the data (Verheul et al., 1999).

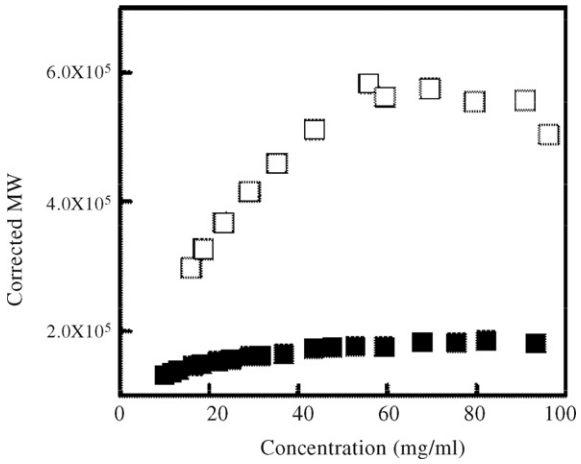


Fig. 5. Corrected average molecular weight of a model monoclonal antibody used by Liu et al. (2005) as a function of protein concentration and salt content. The loading concentration used in the sedimentation equilibrium experiments was 100 mg/ml. The buffer contained 240 mM trehalose, 40 mM histidine, 0.04% polysorbate 20 with no added (open squares) and 150 mM NaCl (solid squares).

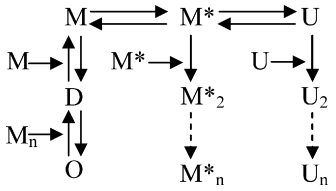
The above-mentioned examples discuss the increases in self-association due to volume occupancy by an inert solute. Conversely, if the process of association is governed by the rate of encounter of the two interacting molecules, i.e. it is diffusion limited, self-association is expected to decrease with increasing concentration of the inert crowding agent (Minton, 2005). However, in high concentration solutions of single protein, the excluded volume effect imposed on the protein by other like molecules can be expected to promote, rather than inhibit, self-association similar to the examples cited above. This will be the case since diffusion of interacting molecules to encounter other like molecules in order to form the association transition state cannot be the rate-limiting step in these solutions due to the presence of excess interacting protein molecules. Verheul et al. (Verheul et al., 1999) have studied the self-association behavior of beta-lactoglobulin as a function of solution pH, ionic strength, and protein concentration by utilizing small-angle neutron scattering. The authors determined the weight average molecular weight of beta-lactoglobulin in solutions of pH 6.9 and 0.1 M NaCl with the protein concentrations ranging from 2 to 104 mg/ml. The solution temperatures studied were 20 and 45 °C. Fig. 4 presents the results from the study in which the ratio of the weight average molecular weight to the monomer molecular weight (M/M_{mono}) is shown as a function of protein concentration. A significant effect of concentration on the self-association induced increase in molecular weight is evident from Fig. 4 at both the temperatures studied.

AUC has also found application in analyzing self-association in concentrated solutions. Due to technical limitations, conventional real-time AUC measurements cannot be performed on concentrated protein solutions. Attri and Minton (1986) developed a microfractionation technique for separating the contents of small centrifugation tubes into 0.1 mm slices followed by their subsequent analysis for concentration. Such a microfractionator is used for post-centrifugation, rather than real-time, analysis of concentrated protein solutions that have been centrifuged in an analytical centrifuge. Subsequently, a concentration gradient profile can be constructed as a function of distance from the meniscus. Darawshe et al. (1993) have described a method to minimize the alteration of the fraction composition during the post-centrifugation fractionation process. Preparative ultracentrifugation as described by Minton (1989) in combination with microfractionation have been used by various researchers (Howlett et al., 2006; Zorrilla et al., 2004; Liu et al., 2005) for analyzing the solution behavior of proteins in concentrated solutions.

Liu et al. (2005) conducted sedimentation equilibrium studies utilizing a preparative ultracentrifuge with a microfractionator to detect self-association in a monoclonal antibody solution at a loading concentration of 100 mg/ml. Initial calculations yielded an apparent molecular weight less than that of an antibody monomer. To investigate this further, the contribution from thermodynamic non-ideality arising due to protein charge and excluded volume were accounted for by the method reported by Chatelier and Minton (1987) The resultant corrected average molecular weight calculations established the presence of self-association in the model protein solutions. The extent of self-association increased with protein concentration and decreased with addition of salt as shown in Fig. 5. Zorrilla et al. (2004) utilized a similar approach to detect non-ideality and self-association in solutions of ribonuclease A, ranging in protein concentration from 1 to 200 mg/ml.

4.1.2. Aggregation

Protein aggregation is regarded as a more serious challenge in protein formulation development as compared to self-association since it is an irreversible process. Protein aggregates could be classified as being either soluble or insoluble. A working definition often employed for soluble aggregates is that they should not be visible to the naked eye and not be retained on a 0.22-μm filter (Cromwell et al., 2006b). Any aggregates bigger in size or visible as particulates are refereed to as insoluble aggregates. The biggest challenge presented by aggregates in pharmaceutical preparation is that of safety since they are known to elicit immunogenic responses (Lobo et al., 2004; Shire et al., 2004; Koren et al., 2002) as observed for aggregates of insulin (Maislos et al., 1988), human growth hormone (Moore and Leppert, 1980), interleukin-2 (Prummer, 1997), interferon-α (Braun et al., 1997), and immunoglobulin (Henney and Ellis, 1968). Immunogenic responses elicited include generation of neutralizing antibodies that challenge the efficacy of the product, hypersensitivity reactions, as well as generation of antibodies that neutralize endogenous proteins. A critical factor governing the generation of an immunogenic response to an aggregate is its molecular weight (Rosenberg, 2006). Particulates and aggregates exceeding 100 kDa with greater than 10–20 ligands per aggregate have



Scheme 1.

been observed to be efficient inducers of immunogenic response whereas smaller aggregates are usually not as efficient. However, it has been observed that large monomeric proteins exceeding 100 kDa in molecular weight are not immunogenic indicating the necessity of aggregate formation in generating an immunogenic response. Frei et al. (1965) have observed that particulate aggregates are more actively phagocytosed by macrophages as compared to other soluble species not removable by ultracentrifugation.

Often a small change in native conformation is enough to result in significant aggregate formation. Aggregation can either be a result of covalent interactions as exemplified by disulfide bridge formation or non-covalent interactions like hydrophobic interactions between the hydrophobic regions of the expanded or unfolded protein molecules.

A generalized mechanism proposed for aggregation (Scheme 1) proceeds via the first order reversible formation of an intermediate species (M^*) from the native protein (M) and its subsequent aggregation (M_n^*) in a higher order process (Kendrick et al., 2002; Lumry and Eyring, 1954). The intermediate species can further denature to the fully unfolded species (U), which can also aggregate (U_n). The symbols D and O in Scheme 1 refer to the reversible formation of a dimer and an oligomer from the native protein molecule through self-association.

The process of aggregation thus involves a minor or a major structural change in the native conformation followed by assembly of these native-like, partially unfolded, or fully unfolded species to form higher molecular weight aggregates. Although a fully unfolded protein molecule has been found to be involved in aggregation (Goldberg et al., 1991; Zettlmeissl et al., 1979), often a relatively minor transition in the tertiary structure can induce significant aggregation as observed for recombinant human factor VIII (Grillo et al., 2001), bovine growth hormone (Brems et al., 1988), granulocyte colony stimulating factor (Krishnan et al., 2002), human interferon-γ (Kendrick et al., 1998b), and equine beta-lactoglobulin (Ikeguchi et al., 1997). Kendrick et al. (1998a) have studied the solution aggregation behavior of recombinant human interferon-γ and found that a 4% increase in molecular diameter (and a consequent 9% increase in surface area) is sufficient to form the aggregation prone intermediate state.

The fact that relatively minor transitions in the native conformation are enough to trigger aggregation, significant aggregate formation can result over time even under those solution conditions in which the native state is greatly favored. In concentrated solutions, this becomes an even bigger concern due to a higher order dependence on protein concentration. Higher protein concentration results in a solution being deemed unstable even under those solution conditions in which a relatively lower concentration solution would have been stable for the duration of its shelf life. This would be especially true for those proteins in which the rate-determining step is the assembly of the aggregation intermediate to form the aggregate and not the generation of the aggregation intermediate. In these systems, PPI are expected to be related to protein aggregation. Ho et al. (2003) have studied the aggregation behavior of denatured and reduced lysozyme in solutions of varying guanidinium hydrochloride (GuHCl) concentrations. The authors also calculated the second virial coefficient of denatured and reduced lysozyme in these solution conditions. The second virial coefficient decreased from $\approx 1.8 \times 10^{-3}$ ml mol/gm^2 at 6.0 M GuHCl to zero at 3.0 M GuHCl. Consistent with this behavior, the authors observed protein aggregation at GuHCl concentrations below 3.0 M and consequently, second virial coefficient could not be measured at these GuHCl concentrations. No aggregation was observed for 6.0 M GuHCl solutions. Studies were also conducted at two different protein concentrations of 1 and 3 mg/ml and 1.25 M GuHCl concentration in order to assess the effect of protein concentration on aggregation at constant second virial coefficient. At this GuHCl concentration, second virial coefficient was predicted to be negative from extrapolation of higher GuHCl concentration data. The authors observed increase in aggregate formation with time at 3 mg/ml protein concentration but not at 1 mg/ml supporting the fact that second virial coefficient was a thermodynamic parameter, which could not simplistically predict the rate of aggregation.

However, pharmaceutical preparations are formulated under solution conditions in which the protein is present in its native conformation. The analysis of PPI is also conducted under the same conditions. However, can PPI determined for the native protein be related to the aggregation behavior of the non-native (structurally altered) protein molecules? The intuitive answer to this question would be "no", if the aggregating species was significantly different in conformation and properties from the native state. This is because the nature of interaction of this structurally altered species with other structurally altered protein molecules would be different from the nature of the interactions between the native molecules. However, for proteins in which the aggregating species is only slightly different in conformation from the native state or is native-like, the nature of PPI of the native molecule could relate to the extent of aggregation (Valente et al., 2005). Krishnan et al. (2002) have studied the aggregation behavior of granulocyte colony stimulating factor stored at pH 6.9 and 37 °C, a condition in which the native state of the protein is favored. The authors conducted conformation and kinetic studies to conclude that it was not necessary to drastically perturb the native structure of the protein to produce aggregates and that the population of the aggregation prone species was one in every six million native protein molecules. In spite of the minor structural change in the native conformation and small population of the aggregation prone species in the native state ensemble, nearly 100% loss of the monomer was observed after 5 days of storage at 37 °C. Chi et al. (2003a) further characterized the PPI in freshly prepared solutions of granulocyte colony stimulating factor under different solution conditions. The authors observed a negative second virial coefficient (-2.3×10^{-3} ml-mol/gm^2) in solution condition (pH 7.0) similar to that in which aggregation was observed for by Krishnan et al. (2002) (pH 6.9). No aggregation was observed at pH 3.5 where the second virial coefficient was positive (13.5×10^{-3} ml-mol/gm^2). Under both these solution conditions, the free energy of unfolding was comparable (11.3 ± 0.71 kcal/mol at pH 3.5 and 9.48 ± 0.49 kcal/mol at pH 7.0) indicating the critical role of PPI in the process of aggregation.

Although the aforementioned studies were conducted under relatively low protein concentrations, they demonstrate the significant role of PPI in governing protein aggregation., In high concentration protein solutions, the role of PPI in affecting protein aggregation is expected to be amplified. Such an expectation would hold unless the higher concentration of protein affects the rate-determining step for the process of aggregation. The rate-determining step for aggregation could change when the presence of a higher number of protein molecules reduces the rate of generation of the aggregation prone species, by stabilizing the more

compact native state, as compared to the rate of protein assembly to form aggregates (Kinjo and Takada, 2002). This has been demonstrated for hemoglobin and fibrinogen by Guo et al. (2006) in a recent work. The authors noted an increase of ≈8 and ≈3 °C in the thermal melting temperature (T_m) for hemoglobin and fibrinogen, respectively, at the higher protein concentration (concentrations of hemoglobin studied were 0.3 and 245 mg/ml and for fibrinogen were 0.17 and 59 mg/ml). However, increase in stability of the native state with increasing protein concentrations is not a rule for protein solutions since exceptions also exist. As demonstrated by the same authors (Guo et al., 2006), T_m decreased by ≈4.5 and ≈20 °C for lysozyme and BSA, respectively, at higher protein concentrations (lysozyme concentrations studied were 0.41 and 350 mg/ml and for BSA were 0.27 and 330 mg/ml).

4.2. Rheology of protein solutions

Einstein, in 1906, first attempted to relate the specific viscosity of a dilute suspension of non-interacting rigid spheres to their volume fraction (Menjivar and Rha, 1980; Shaw, 1992). Since then, attempts have often been made to relate the rheological behavior of a solution to solute's properties. For protein solutions, flow character of the solution depends upon the composition of the system, concentration of the protein and properties of the protein molecule in solution. Information relating the solution environment and the associated flow behavior can be of immense importance in predicting and modifying the rheological behavior and in imparting the desired properties to protein solutions (Tung, 1978). Most protein solutions exhibit non-Newtonian flow whereby the rheological properties show stress/strain dependence and thus the conditions of testing employed for studying the flow behavior require acute attention. The reasons for this kind of non-Newtonian behavior are the PPI that exist in protein solutions. If there were no such interactions, flow properties would depend only on the volume fractions or concentration of the dispersed phase (Lee and Rha, 1979; Kinsella, 1984; Tung, 1978). In such a case, excluded volume will be the primary determinant of solution property and solution flow properties like viscosity will be similar for similarly sized protein molecules. However, in practice significantly different flow profiles have been observed for similarly sized protein molecules or even for the same protein present in different solution environments (Liu et al., 2005). This indicates an involvement of protein and solution environment specific variables in governing protein solution rheology, i.e. involvement of energetic PPI in addition to excluded volume contributions.

4.2.1. Viscoelastic properties of protein solutions

An important aspect of the rheology of macromolecular solutions is that of viscoelasticity, which is studied in oscillatory experiments involving cyclic application of stress or strain. Solution viscoelasticity is intimately linked to the intermolecular interactions and study of solution viscoelasticity can lend an insight into the nature of intermolecular interactions. Viscoelasticity is observed when a viscous liquid exhibits a solid like behavior. Under these conditions, the liquid possesses not only viscous properties but also some elastic properties. On application of a mechanical force, such a system undergoes changes in its structure on a microscopic scale that result in both loss and storage of the applied mechanical energy. The cause for dissipation of energy is the internal friction that exists between the structural elements that constitute the material and material flow, which is a result of relaxation processes. Therefore, the mechanical energy is lost through an irreversible deformation. The cause of energy storage is the inability of certain processes to relax on the timescale of the applied oscillation due to strong interactions between their structural elements. Consequently, some fraction of the applied energy is stored due to reversible deformation (Matuszek, 2001).

Viscoelastic characterization of solutions is conducted using dynamic measurements in which sinusoidal oscillatory strain is applied with an angular frequency (ω) and the induced stress is measured as a function of frequency yielding a value of frequency dependent shear modulus, $G(\omega)$. For viscoelastic materials studied by dynamic experiments, shear modulus is dominated by viscous contributions at low frequencies and elastic at high frequencies. This is because the molecular networks/transient structures tend to disentangle/relax during longer period of oscillations at lower frequencies and the solution behaves more as a viscous liquid. At high frequencies however, the molecular networks/transient structures cannot disentangle/relax during relatively shorter period of oscillations and the system tends to behave more elastically (Nishinari, 1997). Thus, shear modulus for a viscoelastic material is a complex shear modulus comprising of an elastic contribution called the storage modulus, G' (proportional to the energy stored), and a viscous contribution called the loss modulus, G'' (proportional to the energy dissipated) (Aklonis and MacKnight, 1983).

On the molecular level, these moduli can be related to the average relaxation or rearrangement time (τ) for molecular motions as follows (Macosko, 1994; Ferry, 1980)

$$G' \propto \frac{\omega^2\tau^2}{1+\omega^2\tau^2} \tag{6}$$

$$G'' \propto \frac{\omega\tau}{1+\omega^2\tau^2} \tag{7}$$

When a liquid is subjected to a steady shear flow, a transient order accompanied by decrease in entropy is generated in the liquid. The decrease in entropy is associated with the storage of applied energy and the preferred orientation effect is opposed by Brownian motion, which tends to produce a distribution of orientations. Such a preferred orientation transforms to a random orientation with an exponential time decay, i.e. $e^{-t/\tau}$, where τ, as discussed above, represents the relaxation time for that specific mode of motion, i.e. rotational, translational, etc. (Ferry, 1980). Although, it is not possible to determine these individual relaxation times for different kinds of molecular movements, an average measure of these relaxation times or the most probable relaxation time can be obtained. In the case of oscillatory flow, the frequency of strain application becomes a critical parameter governing the magnitude of both storage and loss moduli. For frequencies at which $\tau \ll 1/\omega$, enough time is allowed for the molecules to undergo relaxation and reach a random distribution of orientations so that the extent of energy stored is minimal and the applied energy is lost as the liquid flows. The liquid in such a case behaves as a viscous fluid with no fraction of energy being stored. For frequencies at which $\tau \gg 1/\omega$, the relaxation to random orientation cannot occur on the timescale of single oscillation and the applied energy gets stored in the system instead of being dissipated by means of flow or reorientation. The liquid in such a case behaves as a rigid or elastic material with minimal dissipative energy loss and the storage modulus approaches a constant high-frequency value. With measurement frequency being such that $\omega\tau \to 1$ or $\omega \to 1/\tau$, the liquid stores part of the applied energy and dissipates the rest and thus qualifies as a viscoelastic liquid. Since solvent molecules relax much faster than solute molecules especially when the solute molecules are polymeric or macromolecular in nature, they do not contribute significantly to G' unless the frequency is increased to GHz range (Endo, 1979).

Solutions of native globular protein molecules have not been extensively studied for viscoelastic behavior since they do not exhibit appreciable viscoelastic character up to frequency of 200 Hz,

which is the upper limit of conventional rheometers. This is because the time scale of oscillation (inverse of the measurement frequency) during measurements is large as compared to τ of PPI and the energy storage by protein solution is not possible. For analyzing viscoelastic behavior of native protein solutions, higher frequencies on the high kilohertz (kHz) to megahertz (MHz) range need to be employed so that the timescale of oscillation is of the order of the relaxation time of PPI in these solutions. Rheometers based on piezoelectric quartz sensors and operating at MHz frequencies (Mason et al., 1949; Kudryashov et al., 2001; Kanazawa and Gordon, 1985) have been developed for analysis of solution rheology although their application for understanding native protein solution behavior has remained largely unexplored until very recently. Saluja and Kalonia (2004) have recently developed a small volume ultrasonic shear rheometer capable of operating at MHz frequencies, which requires only 10–20 μl of test sample. The authors have utilized the developed rheometer specifically for understanding the nature of PPI in high protein concentration solutions (Saluja et al., 2006, 2007; Saluja and Kalonia, 2005).

4.2.2. Factors governing protein solution rheology

Rheological behavior of protein dispersions is intimately linked to the characteristics of the protein itself as well as the environmental factors. The characteristic properties of the protein that are important in this aspect are its conformation, shape, size, solubility, swellability, and charge which are influenced by environmental conditions like temperature, concentration, pH and ionic strength (Hermansson, 1972; Hermansson and Akesson, 1975b).

4.2.2.1. Intrinsic viscosity and hydrodynamics. Viscosity of protein solutions is largely dependent on the hydrodynamic behavior of the protein molecules in solution and is a function of PPI between adjacent molecules and their spatial distribution. The problem of relating viscosity of the colloidal dispersions with the fundamental nature of the dispersed particles has been the subject of much experimental investigations and theoretical considerations (Harding, 1980, 1981; Lundqvist, 1999). Such an effect is defined in terms of some viscosity functions such as relative (η_{rel}), specific (η_{sp}) and reduced or reduced specific viscosity (η_{red}).

$$\eta_{rel} = \frac{\eta}{\eta_0} \tag{8}$$

$$\eta_{sp} = \eta_{rel} - 1 = \frac{\eta - \eta_0}{\eta_0} \tag{9}$$

$$\eta_{red} = \frac{\eta_{sp}}{c} = \frac{(\eta_{rel} - 1)}{c} \tag{10}$$

where η is the viscosity of the solution of concentration c and η_0 is the viscosity of the solvent. Intrinsic viscosity $[\eta]$ is an intrinsic function of the dissolved/dispersed macromolecule and is defined as the limiting value, at zero concentration, of relative viscosity increment.

$$[\eta] = \lim_{c\to 0}\eta_{red} = \frac{1}{\eta_0}\lim_{c\to 0}\frac{\eta - \eta_0}{c} = \frac{1}{\eta_0}\lim_{c\to 0}\frac{d\eta}{dc} \tag{11}$$

Two factors that contribute to this characteristic property of the dispersed particle are particle shape and size/volume as summarized by the following relationship

$$[\eta] = \nu V_s \tag{12}$$

where ν is the molecular shape factor known as the viscosity increment or universal shape function (Yang, 1961) and V_s is known as the swollen specific volume and is a measure of the solvent associated with the macromolecule. It is defined as the volume of the macromolecule in solution per unit anhydrous mass of macromolecule. Intrinsic viscosity is thus indirectly dependent on axial ratio of the molecule that governs its shape. It is found to increase with the increase in axial ratio and is therefore minimum for a sphere (Tanford and Buzzell, 1956). Because of such dependence, intrinsic viscosity has been used as a measure of the hydrodynamic volume and molecular shape. Change in intrinsic viscosity or in reduced viscosity at low concentration has been used as a measure of the extent of denaturation, which also reflects the relationship with changing axial ratio (Suryaprakash and Prakash, 2000; Ahmad and Salahuddin, 1976). Bull (1940), Booth (1950), and Tanford and Buzzell (1956) have done pioneering work in this field.

4.2.2.2. Solute concentration. Solute concentration has a pronounced effect on the behavior of protein dispersions. In a dilute solution, the total viscosity effect is sum total of the effect caused by each particle and, is a function of the viscosity increment and swollen specific volume. As the concentration increases, deviation from Newtonian behavior becomes more pronounced and a greater resistance to flow is offered.

Mooney (1951) modeled the viscosity of suspensions of spherical solutes taking into account the excluded volume contribution of the solute molecules present at a finite concentration. The excluded volume contribution due to molecular volume was referred to as the crowding effect of solute molecules. The following expression for the relative viscosity was proposed by Mooney

$$\eta_{rel} = \exp\left(\frac{S\phi}{1 - k\phi}\right) \tag{13}$$

where S is the universal shape factor and k is the crowding factor. A good fit of the proposed model to the viscosity data of spheres obtained by Vand (1948) was obtained using a value of 2.5 for S and 1.43 for k.

De Gennes (1979) introduced the concept of critical concentration, c^*, in connection with the behavior of coil shaped molecules. As the concentration exceeds this critical concentration value, molecular domains begin to overlap and viscosity dependence on concentration begins to increase. Different authors have proposed different equations that relate c^* to chain dimension, intrinsic viscosity, molecular mass and radius of gyration. It is usually found to be proportional to M/R_g^3, where M is the molecular mass and R_g is the radius of gyration. Intrinsic viscosity is proportional to R_g^3/M thus, making critical concentration inversely dependent on intrinsic viscosity (Papanagopoulos and Dondos, 1995). This overlap in molecular flow domains explains the increase in apparent viscosity of macromolecular dispersions with increase in concentration as predicted by the rheological volume concentration theory by Frisch and Simha (1956). In-depth work is reported in literature whereby the effect of concentration has been analyzed on flow behavior (Pradispasena and Rha, 1977; Monkos, 2000; Gill and Tung, 1976; Ehninger and Pratt, 1974).

Wagner et al. (1992) measured apparent viscosity of soy protein isolates at concentrations ranging from 1.6% to 14.0% and reported an increase in the shear stress with concentration over the entire range of strain rates measured due to higher amount of water that could be immobilized. The effect of concentration on apparent viscosity was more profound for partially denatured soy protein isolates, which would be expected due to greater PPI as a result of expansion of the protein coil. Menjivar and Rha (1980) studied the effect of concentration and shear rate on zein dispersions in 95% ethanol. The authors observed a consistent increase in the shear viscosity of dispersions with increase in concentration with a discontinuous increase in the slope of the viscosity versus concentration plot at 42% solute concentration. The effect of shear rate was to decrease the apparent viscosity, which is expected because increasing shear rates tend to align the molecules with the shear plane so that the frictional resistance is reduced. As a result, a

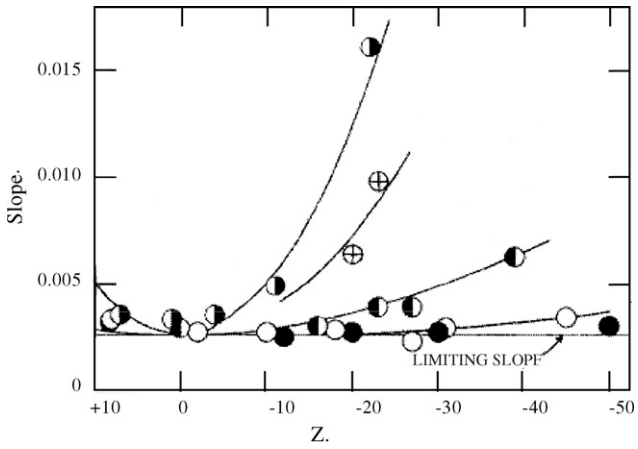


Fig. 6. Concentration dependence of reduced viscosity, i.e. "slope" or $d\eta_{red}/dc$ as a function of net protein charge for solutions of bovine serum albumin at different ionic strengths. Ionic strength was maintained with potassium chloride. The symbols represent solution ionic strengths of 0.01 M (open/solid circle), 0.02 M (positive circle), 0.05 M (solid/open circle), 0.15 M (open circle), and 0.50 M (solid circle). The change in net protein charge was due to chloride binding (Tanford and Buzzell, 1956).

random structure at low shear rates changes to a shear-oriented structure at high shear rates. Although all the above examples demonstrate an increase in rheological parameters with protein concentration, the increase is strongly dependent on the nature of intermolecular interactions (Saluja et al., 2006).

4.2.2.3. Electrostatic contribution: effect of pH and ionic strength. Solution pH and ionic strength have a marked effect on the hydrodynamic properties of the protein and therefore, on the flow behavior of the system. This effect is mediated through the effect on charge associated with the protein molecule, its folding and unfolding kinetics, and the solubility of the protein that changes with the pH. Besides shape, volume and hydration factors, electrostatic charge effects can have significant influence on the intrinsic viscosity of a molecule in solution especially if the molecule is multiply charged, i.e. a polyelectrolyte, as proteins usually are. In theory three such contributions have been summarized for a compact globular protein molecule: a 'primary effect' due to the diffuse double layer surrounding the protein molecule, a 'secondary effect' due to repulsion between the double layers of different molecules and a 'tertiary effect' arising from the intermolecular repulsions that affect the shape of the molecule. These three have been collectively referred to as 'electroviscous' effects. Under conditions of high net charge on the protein molecule, electroviscous effect contributes significantly to solution flow behavior. However, under conditions of low molecular charge and/or high charge screening, as is the case with high ionic strength solutions, electroviscous effects are not very prominent and shape, volume, and short range attractive contributions predominate.

Tanford and Buzzell (1956) have conducted experiments on BSA in the pH range of 3.0–10.5. Between pH ≈4.3 and ≈10.5, albumin molecule behaves essentially as an unexpandable molecule. The shape/volume of the BSA molecules in this pH range was not significantly different. Below pH 4.3 and above pH ≈10.5, BSA molecules existed in a relatively expanded form. Thus, in the pH range of 4.3–10.5, the contribution of protein charge to solution viscosity, without any shape/volume effects, could be examined. Fig. 6 shows the results for the change in concentration dependence of reduced viscosity, i.e. $d\eta_{red}/dc$ as a function of net protein charge for solutions of different ionic strengths maintained with potassium chloride. The symbols represent solution ionic strengths of 0.01 M (open/solid circle), 0.02 M (positive circle), 0.05 M (solid/open circle), 0.15 M (open circle), and 0.50 M (solid circle). The change in net protein charge was due to chloride binding. Under conditions of low molecular net charge, at various solution ionic strengths, the $d\eta_{red}/dc$ approached a limiting value (≈26 ml^2/g^2) representing the effect of non-energetic PPI, i.e. shape and volume contributions. The increase in $d\eta_{red}/dc$ above this limiting value was greatest at low ionic strength and high net charge as exemplified by 0.01 M ionic strength data in the figure. At high ionic strengths, even a high net protein charge did not result in a significant increase above the limiting value due to screening of charge-charge interactions (e.g. data for ionic strengths 0.15 and 0.50 M, respectively). Similar results were obtained when protein charge was varied by changing the solution pH.

Buzzell and Tanford (1956) have conducted experiments for determining the effect of charge and ionic strength on solution viscosity of ribonuclease, another protein that is known to maintain a compact conformation in a broad pH range. Results similar to those obtained by Tanford and Buzzell (1956) for BSA have been reported by the authors. In this case, the limiting value of $d\eta_{red}/dc$ was found to be ≈21 ml^2/g^2. Another effect of pH on viscosity is through protein solubility. Van Megen (1974) studied promine D solubility as a function of pH and concluded that the increase in viscosity at pH values away from p*I* was due to an increased solubility of the protein at those pH values.

4.2.2.4. Short-range interactions. An important consequence of increasing solute concentration is the decrease in the intermolecular center–center distance. The intermolecular distance can be calculated as the inverse cube root of the solute number density or the number of solute molecules present per unit volume of the solution. Since proteins are large molecules, the surface–surface distance can be considerably less than the center–center distance depending on the shape and size of the molecules. Because of a decreasing intermolecular distance, the relative contribution of different interactions discussed in the previous sections to the overall PPI would change with protein concentration. This would happen since certain interactions only become dominant at short separation distance (e.g. van der Waals, dipolar interactions) whereas others are relatively longer range interactions (e.g. charge–charge interactions). A recent report details the rheological analysis conducted on a monoclonal antibody (IgG_2) in moderate to high protein concentration conditions. The center–center distance in 20 mg/ml antibody solution (molecular weight ~ 144,000) is ~23.0 nm, which corresponds to a surface–surface distance of ~12.0 nm assuming the IgG_2 molecule to be a sphere with hydrodynamic diameter of ~11.0 nm. Thus, at this concentration the surface-surface distance and hydrodynamic diameter are comparable. At 120 mg/ml (the highest protein concentration used by the authors), the center–center distance reduces to ~12.0 nm resulting in a surface–surface separation of ~1.0 nm, which is 1/11th of the molecular hydrodynamic diameter. The results of the work (Saluja et al., 2007) are presented in Fig. 7 in which the variation in solution G' is shown as a function of protein concentration and solution pH. Solutions at protein concentration below 80 mg/ml showed the greatest G' at pH 3.0 whereas at higher concentrations, pH 7.4 and 9.0 solutions exhibited sharp increase in solution G'. This kind of behavior indicated that at pH 3.0 long-range interactions dominated since these solutions exhibited higher solution G' at lower concentrations. However, at pH 7.4 and 9.0, short-range interactions dominated solution G' since these solutions exhibited higher solution G' at higher concentrations, i.e. when intermolecular distances were smaller.

The change in relative contribution from different interactions, with changing protein concentration and consequently intermolecular distance, also has implications for understanding of protein

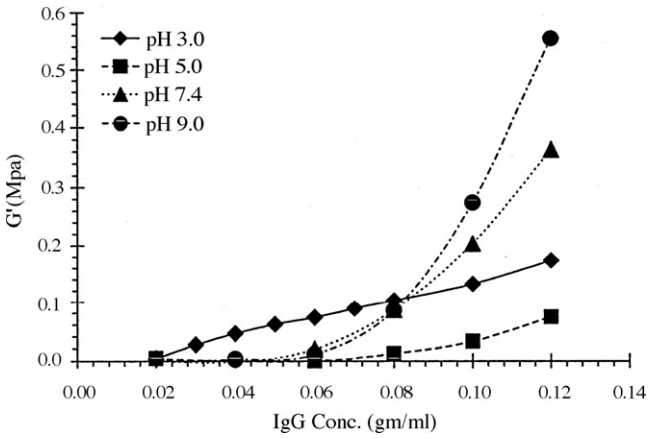


Fig. 7. Solution storage modulus (G') for IgG_2 solutions at a frequency of 10 MHz measured using the ultrasonic shear rheometer as a function of IgG_2 concentration at different solution pH (Saluja et al., 2007).

solution behavior at high protein concentrations from relatively dilute solution analysis data. Due to a dearth of analytical techniques for studying concentrated protein solutions, a commonly employed approach is to predict the behavior of proteins in concentrated solutions from dilute solution measurements. Although such an approach has its merits especially due to availability of various methodologies for dilute solution analysis and since it requires lesser amount of protein sample, in certain cases it can fail to predict the effect of a solution variable on protein behavior in concentrated solutions. Fig. 8 shows one such example for an IgG_2 antibody. The figure compares the effect of solution ionic strength on interaction parameter, k_D, measured by dynamic light scattering for dilute protein solutions (protein concentration 4–12 mg/ml) with solution G' measured at 120 mg/ml on ultrasonic shear rheometer (Saluja et al., 2007). Both measurements indicated a decrease in repulsive PPI with increasing ionic strength up to an ionic strength of ~40 mM. Beyond 40 mM, the effect of ionic strength on dilute solution parameter k_D was insignificant indicating no change in PPI with ionic strength. However, solution G' continued to decrease at ionic strengths greater than 40 mM. The results thus indicated a distinct difference in protein behavior in concentrated solutions as compared to dilute solutions. This observation demonstrates that caution must be exercised when extrapolating protein solution behavior in concentrated solutions from dilute solutions, particularly in instances where the effect of a solution variable on protein behavior in dilute solution is not evident.

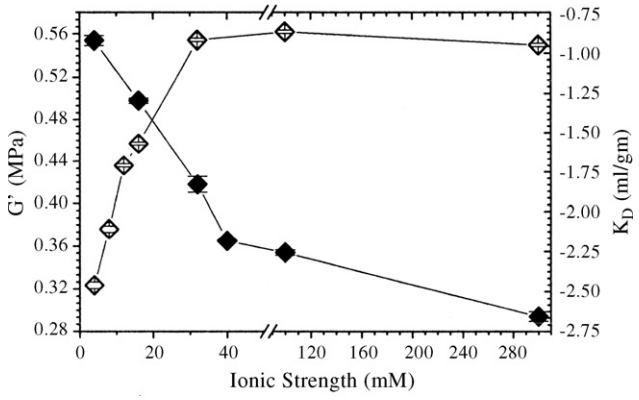


Fig. 8. Solution G' (solid diamonds), at 120 mg/ml IgG_2, and k_D values (open diamonds), calculated from relatively dilute solution measurements (4–12 mg/ml IgG_2), for IgG_2 solutions as a function of solution ionic strength (Saluja et al., 2007).

4.2.2.5. Protein self-association and solution rheology. Proteins often undergo self-association reactions in both physiological and pharmaceutical systems, a process that is intimately linked with the rheology of a solution. Self-association can bring about change in solution rheology and on the contrary, the process of self-association can be affected by a change in solution rheology. For diffusion-controlled reactions, the rate of self-association decreases with an increase in solution viscosity brought about by addition of an inert excipient, since the rate of diffusion decreases with increasing solution viscosity (Schreiber, 2002). In absence of a viscosity-enhancing agent, high concentration solutions of proteins having a tendency to self-associate can however result in increased solution viscosity. This is usually observed in biological systems in which self-association of proteins is involved in hyperviscosity syndrome, a condition associated with increased viscosity of physiological fluids (Pope et al., 1975). Hall and Abraham (1984) have investigated the reversible concentration dependent polymerization behavior of IgG_1-λ myeloma protein involved in hyperviscosity syndrome of the serum. A predominant contribution of dispersion and hydrophobic forces in driving the self-association and subsequent polymerization of the model protein has been reported by the authors. Senderoff et al. (1998) have observed a dramatic change in solution viscosity for glucagon-like peptide 1 in increasing peptide concentration from 5 mg/ml, where the solution exists as a liquid, to 25 mg/ml when a gel like system is formed. The authors have attributed the increase in solution viscosity to self-association of glucagon-like peptide 1.

In pharmaceutical protein solutions as well, a correlation between self-association and solution viscosity of high concentration protein solutions has recently been reported by Liu et al. (2005). The authors investigated three humanized monoclonal antibodies constructed from the same IgG_1 framework with κ light chains and differing only in their complementarity determining regions. The solutions were analyzed up to a concentration of 130 mg/ml in low ionic strength buffers containing 266 mM sucrose, 16 mM histidine, and 0.03% polysorbate 20 at pH 6.0. One of the three antibody solutions (Ab1) exhibited viscosity of ≈80 centipoise (cP) at 25 °C. The viscosity was highly dependent on antibody concentration and concentrated solutions exhibited shear thinning. The solution viscosities for the other two antibodies were ≈5 cP at 130 mg/ml. Self-association of Ab1 was found to be the cause for the relatively high solution viscosity. Addition of 200 mM sodium chloride disrupted self-association and resulted in decrease of solution viscosity to ≈10 cP at the same concentration. The involvement of soluble aggregates in affecting solution viscosity was also assessed by quantitating the soluble aggregates after lyophilization as well as after storing samples at 60 °C and measuring solution viscosity. No correlation between the amount of soluble aggregate and solution viscosity was observed. Kanai et al. (2008) extended the analysis of Ab1 solutions to elucidate the mechanism of self-association. The authors concluded that self-association operated predominantly through Fab–Fab interactions and that chaotropic salts were more effective in reducing solution viscosity as compared to kosmotopic salts.

5. Summary

Concentrated and crowded protein solutions are frequently encountered in the field of pharmaceutical product development as well as in physiological systems. The behavior of these solutions can be markedly different from dilute solutions. The primary reason for this effect of solute concentration on solution behavior is the intermolecular interactions between the protein molecules, which alter the effective protein concentration or its thermody-

namic activity in these non-ideal solutions. A good measure of non-ideality of a solution is provided by analyzing the virial coefficients that govern the dependence of solute's thermodynamic activity or a colligative property like osmotic pressure on its concentration and consequently determine the nature and extent of PPI. Various types of interactions and forces contribute to the overall PPI in high concentration protein solutions and include but are not restricted to excluded volume effects, electrostatic charge–charge and dipole interactions, van der Waal's forces, effects due to salt binding, hydration, and exclusion, and contributions from protein self-association. Attempts have been made to understand the protein behavior in solution based on repulsive charge-charge and attractive van der Waal's forces by incorporating the DLVO model for interactions of colloidal particles. However, other forces often have to be included in order to explain the observed protein behavior especially in concentrated protein solutions. In these solutions, intermolecular center–center distances become small, the number of interacting molecules becomes large, and deviation in solution properties from a predicted path affects both short-term and long-term behavior of the solution.

From a pharmaceutical perspective, the two more critical consequences of strong PPI in concentrated solutions are protein association and aggregation and the alteration in solution flow properties, i.e. its rheology. Self-association and aggregation in protein solutions can potentially limit the formulation shelf life as well as raise concerns with regard to safety, efficacy and visual appearance of the final product. Immunogenecity of these higher molecular weight species challenges the safety of the product. Self-association, a reversible formation of higher molecular weight oligomers, is usually of a less severe consequence in protein formulations. However, slowly dissociating reversible oligomers can have prolonged clearance half-lives and be immunogenic especially when administered by subcutaneous route. They may also promote the formation of irreversible aggregates. Protein aggregates however present a more severe safety challenge as compared to reversible oligomers and trigger a stronger immunogenic response *in vivo*. Since a structural change and a subsequent assembly of the protein molecule is required to form an aggregate, either of these processes could be rate-limiting although usually it is the assembly step and aggregation reactions are often second order in solute concentration. Consequently, it is an even bigger concern for high concentration protein solutions as compared to dilute protein solutions and is intimately linked to the nature of PPI in these solutions. Since minor transitions in the native conformation can result in appreciable amount of aggregate formation over time, it alludes to the fact that rate of aggregation could be related to the nature of PPI between the native protein molecules as long as generation of the aggregation prone species is not rate-limiting.

Solution rheology plays a significant role in governing manufacture, processing, pumping, filtration, and administration of the parental products through syringes. High viscosity observed for some of the novel high concentration monoclonal antibody solutions can potentially result in financial concerns due to loss of unrecoverable product from production and processing vessels in addition to issues during the above-mentioned processes. The key factors underlying the rheology of these solutions include intrinsic viscosity of the protein molecule, protein concentration and occupied volume, electrostatic contribution, short-range interactions, and protein self-association. Any of these factors can play a dominant role in governing solution rheology depending on the protein molecule in solutions and the solution conditions. An important aspect of high protein concentration solutions is their viscoelasticity, which can provide valuable information regarding intermolecular interactions. However, the frequency of analysis is a critical parameter for utilizing solution viscoelasticity for studying PPI. Ultrasonic rheometers operating at megahertz frequencies can prove useful in this regard.

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