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### Minireview: Protein Interactions

Jessica Child

*University of New Hampshire - Main Campus*

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## Protein Interactions: Brief Summary

Jessica Child

Recombinant DNA (rDNA) and monoclonal antibody (MAb) technologies are now being commercialized as therapeutic proteins for treatment of many diseases, from cancer to autoimmune disease. These technologies, however, require new formulations of medications to combat various complications. Intravenous (IV) delivery is one method used in medicine. This method allows great bioavailability, greater control in clinical use, faster pharmaceutical development, and large dose volumes compared to subcutaneous (SC) delivery methods. SC delivery has many other practical advantages: it can be used more frequently and chronically as the dose volume is small, allows easy at-home administration, and could lead to improved compliance of administration from patients. SC injectable products could also enhance the pharmaceutical market by developing new drugs. However, with SC injection, < 1.5 mL can be given per injection. Because most treatments have high dosing (~100 mg per dose), concentrations of 100 mg/mL or greater would be required for SC delivery.<sup>1</sup>

At this concentration, protein-protein interactions resulting in high viscosity, reversible and irreversible aggregation, and molecular crowding are likely to occur, making solubility, stability, delivery, and manufacturing a major concern and barrier to development of SC drugs.<sup>2</sup>

Principles governing protein solubility are more complex than those for small synthetic molecules, making high protein solutions difficult to work with and practically use. Dependence of solubility on ionic strength, salt form, pH, temperature, and certain excipients can be explained by changes in bulk water surface tension and protein binding to ions and water vs. self-association. Changes due to excipients or salt are influenced by changes in conformation or masking of certain amino acids involved in self-association. Protein solubility can be altered by preferential hydration and stabilization (compact conformations). Protein solubility depends on purity, requiring selection of protein formulation to be verified with large scale preparations before use.<sup>1</sup>

Stability of a protein can change greatly when placed in a high concentration solution. Chemical degradation from deamidation, aspartate isomerization, oxidation, and peptide bond hydrolysis can occur. These damages are hydrolytically driven and generally depend on low density kinetics. Degradation from aggregation also occurs in high concentration systems. These damages often result

from bi-molecular collision and can result in covalent or non-covalent associations which may be reversible or irreversible, damaging the contents of a protein drug. If stability of a drug is compromised, it will not be effective or have a practical shelf life.<sup>1</sup>

Aggregation of proteins can impact protein activity, pharmacokinetics, and safety. Irreversible damages are a major problem in drug manufacturing. Reversible aggregation can be overlooked in dilute protein products, though at higher concentrations, the equilibrium can be pushed toward more aggregate formation due to molecular crowding which could cause potential problems. As the concentration increases, the total volume occupied by the protein also increases, decreasing the effective volume available to the protein, yielding a higher apparent concentration and favoring self-association. This also increases the apparent thermodynamic association constant and could be shelf-life limiting.<sup>1</sup>

Molecular crowding effects can also impact physical properties such as viscosity. This is a major factor when it comes to manufacturing high concentration protein products and for administration via injection.<sup>1</sup>

Many experimental and modeling studies have been and continue to be investigated to help understand protein interactions at high concentration. These studies could be useful in manufacturing and preparing SC drug delivery systems in the future.

#### Experimental Findings:

In 1997, Monkos studied the viscosity of hen egg-white lysozyme at a wide range of concentrations and temperatures. He found the viscosity-temperature dependence can be quantitatively described by Arrhenius formula at each fixed concentration. He used the generalized Arrhenius formula to calculate the parameters of Mooney approximation and, by applying an asymptotic form of the generalized Arrhenius formula, intrinsic viscosity and Huggins coefficient could be calculated. It was concluded lysozyme molecules in aqueous solution have hard quasi-spherical particles. Monkos found that as temperature increases, viscosity decreases as the Huggins coefficient increases. Additionally, the activation energy of a protein increased as concentration increased.<sup>3</sup> This suggested large viscosity issues when concentrating proteins into a solution to be injected into a 37°C body system. In this

situation, proteins are concentrated and temperature raised, making the protein solution very viscous and likely unable to flow through a needle.

Zydney et. al (1998) investigated the transport of proteins through porous membranes to look at the effects of colloidal interactions. Membrane transport traditionally considered only steric interactions between solute and pores. Experimental work clearly demonstrated the importance of longer-range colloidal interactions on the rate of solute transport. These properties can greatly affect membrane performance. It was found transport through a porous membrane can be controlled by operating at the pI of a protein in question. It is possible to exploit differences in van der Waals interactions in membrane systems based on the hydrophilic/hydrophobic character of different proteins. Steric, electrostatic, and van der Waals interactions in membrane systems is analogous to the use of the same intermolecular interactions to affect size-exclusion, ion exchange, and reverse-phase chromatography. Future progress in the development of highly selective membrane processes will require application of colloidal interaction theories. Future membranes will likely involve polymers with carefully chosen surface charge and hydrophilicity and buffer conditions chosen to exploit the full range of colloidal interactions that govern protein transport.<sup>4</sup> Understanding protein transport would aid development of drug delivery systems.

Beretta et al. (2000) investigated short-range intermolecular attractive interactions induced by the addition of salt and nonadsorbing polymers. Adhesive-hard-sphere (AHS) potential energy can be used to rationalize phase diagrams for proteins<sup>5</sup>, estimated as quasi-spherical particles as described by Monkos<sup>3</sup>. Solubility and second virial coefficient have been correlated with protein-protein interactions that lead to crystal arrangement and helps determine solubility. Beretta's study tried to single out the most important features of protein-protein interactions through the use of simple van der Waals-electrostatic model that accounts for non-specific interactions with a mean field treatment. The protein is imagined as a hard dielectric sphere with uniform charge distribution based in the DLVO Potential, a combination of short-range attractive interaction and a screened Coulomb repulsion. Short-range attractive interactions are known as the Hamaker potential. At distances shorter than one nanometer the repulsion between individual atoms of approaching molecules must be considered. A reduction of the attractive van der Waals attraction at very short distances arises from a displacement of the van der Waals surface from the charged protein surface. Electrostatic interaction is described by Debye-Huckel screened Coulomb potential. This study characterized protein-protein interactions by measuring the

steepness of the dependence of the mutual diffusion coefficient on the ionic strength. Different proteins have different interaction properties, even with similar shape and size, due to large condensation of positively hydrated counterions on some proteins vs. others. Large repulsive hydration forces show up as a smaller effective van der Waals attractive interaction.<sup>5</sup> This molecular understanding of protein interactions lead to further investigation into various types of molecular interactions that may result in the increased viscosity of protein solutions seen under high concentration.

Kanai et al found reversible self-association of monoclonal antibody in high concentration formulas to result in solutions with high viscosity<sup>2</sup>. It was also found that this viscosity could be reduced with chaotropic anions. Chaotropic salts were more successful in decreasing viscosity than kosmotropic anions. This observation could be explained by Hofmeister series and the net charge of the antibodies used in this study<sup>2</sup>. Additionally, as the secondary and tertiary structure of the MAb was not altered, but guanidine HCl, a monovalent cation, reduced viscosity much more than neutral urea, it was suggested a charge effect may be a more important factor than the chaotropic nature for decreasing the viscosity of a protein solution. It is believed this is done by breaking network self-association of a MAb. Additionally, Fab was found to be primary site of network self-association within a MAb solution<sup>2</sup>. This study not only showed that the problem of viscosity in high concentration solutions could be countered, it also showed that certain areas of proteins could be responsible for the aggregation that contributes to increased viscosity. Both of these finding were directly relatable to the development of SC drugs.

The influences of various properties on protein interactions needed to be investigated to investigate how proteins could be concentrated but remain fluid enough to be pushed through a needle.

Ellipsometry can be used to investigate the influence of ionic strength ( $I$ ) and pH on the adsorption of different proteins onto preabsorbed layers of different polycations. Silva used this technique in comparison of three polycations showed hydrophobic interactions. Comparison between proteins with similar isoelectric points ( $pI$ ), BSA and BLG, indicated the importance of protein charge anisotropy. With pH close to  $pI$ , ionic strength dependence of the adsorbed amount of protein corresponded to Debye lengths close to the protein radius. Visualization of the protein charge suggested ionic strength conditions correspond to suppression of long-range repulsion between polycations and protein positive domains without diminishing of short-range attraction between polycation segments and locally negative protein domains. This is consistent with the disappearance of the adsorbance maxima at pH

above or below pI. In the former case, adsorption amount of the protein decrease exponentially with  $I^{1/2}$  due to screening of attractions. In the latter case, adsorption of proteins decreased at low  $I$  due to string repulsion. Close to or below pI proteins adsorbed more strongly to polycations with long linear aliphatic carbon chains than to short aliphatic carbon chains or an ammonium functional group. This is most likely due to hydrophobic interactions with the long alkyl chain (apolar side chains). Above pI, the adsorption was stronger to the ammonium functional group because the chains could assume more loosely bound layers due to lower linear charge density (forming loops or tails). This experiment demonstrated protein interactions in ionic conditions and showed protein side chain can affect interactions.<sup>6</sup> This study was important for the development of high concentration protein systems because it showed using certain proteins could decrease interactions, aggregation, and therefore viscosity of a solution.

Galush (2012) showed that the high viscosity characteristic of high protein concentration solutions is caused by molecular crowding and direct interactions among proteins. The equation  $\text{Log}(\eta_{\text{mix}}) = x_1 (\log \eta_1) + x_2 (\log \eta_2)$  describes the viscosity of a mixture, where  $\eta_i$  is viscosity of a protein solution  $i$  at total mixture concentration and  $x_i$  is the weight proportion of protein  $i$  in the final mixture. This equation provides good representation of a mixture as long as each protein in system is characterized on its own. Interactions are not strongly dependent on electrolyte conditions or excipient-protein interactions in protein blend systems. Using volume, weight, or molecular proportions in protein mixtures yields the same results.<sup>7</sup> This is important to understand and can be used when investigating what the viscosity of a protein mixture may be while developing new formulations.

#### Models:

Being able to accurately simulate protein interactions is important to be able to make predictions about which experiments maybe plausible, rather than wasting materials and resources. Attempting simulation can also lead to a deeper understanding of molecular interactions.

In 1996, Agera et al used the UNIQUAC model to investigate the activity coefficient of proteins. The UNIQUAC model is studied relative to UNIFAC model, which enhances UNIQUAC by group contribution approach to allow extension of the model to other systems. Experimental osmotic pressure measures provided activity coefficient data for comparison to this model. Activity coefficients were then used to

determine solution protein solubility. This model allowed prediction of the effects of salt concentration, pH, salt type, and temperature on protein activity coefficients and protein solubility. Binary systems were used involving a protein and pseudosolvent. Properties of water were used for the pseudosolvent. A new parameter of molar composition resulted from this work and supported the prediction of protein activity coefficient in terms of component type, composition, and temperature. This model matched experimental data with a deviation of only 0.54% and showed the original UNIQUAC model is applicable to protein-salt-water solutions. A relationship introduced between activity coefficient and protein solubility that allowed a qualitative interpretation of protein solubility with respect to salt concentration, type, pH, and temperature.<sup>8</sup> Because protein solubility at high concentrations is a barrier to the development of a successful SC drug delivery system, this model can be applied to the development of new drugs.

Roth et al attempted to find calculations to account for geometric irregularity of protein molecules and material properties of interacting media. Van der Waals attractive forces stood out over most conditions and their strength was found to increase sharply as intervening distances between molecules decreased. Van der Waals interaction of atoms comprise of the sum of Keesom, Debye, and London contributions. Analyses of van der Waals interactions in colloidal bodies use the Hamaker approach or a variation. Microscopic and macroscopic approaches have been used to determine Hamaker constants. Microscopic methods relate Hamaker constant to molecular properties such as ionization potential. Macroscopic approaches include utilization of the Lifshitz theory which used the dielectric spectra and geometry when looking at protein interactions. Modeling van der Waals requires approximating geometry of the bodies and determining Hamaker constant. The assumed shape of a sphere is commonly used for analytical expressions. This work found that generally accepted estimates of the magnitude of van der Waals interactions involving proteins were much higher than the true values. This had important implications for analysis of protein behavior in solution near surfaces, such as the walls of a needle during injection. Orientational distributions of dispersion interaction energies relative to Hamaker constant were discovered. 1. Protein-surface interactions energies at 1 Å can be represented by log-normal distributions with no correlation with protein size, 2. protein-protein interactions show tails that are typically longer, with small number of highly favorable orientations observed showing a less successful fit, and 3. not only does the sphere model fail to capture distribution energies in respect to orientation, it also shows a poor estimation of magnitude of van der Waals interactions and does not scale accurately with size or molecular weight. Comparison of globular proteins interacting at 1 Å

nearest separation from planar surface showed spherical assumption overestimates magnitude of average van der Waals interaction energy and correlation of average protein-surface van der Waals energy with respect to molecular volume suggests universal curve can be used to approximate the magnitude of protein-surface interactions and protein-protein interactions. Detailed shape effects appear to be less important for proteins with larger charges; however the shape is influential for small charges. Most favorable interactions are fairly close to sphere results. For protein-protein interactions, a shift from the upper to lower side of the range of energies for all orientations studied was seen as the gap distance decreases; this effect was not seen in protein-surface interactions. Lifshitz gave a rigorous basis for calculating Hamaker constants within a continuum framework and values depended strongly on accuracy and the amount of detail in the representation used of the spectral data. This work used spectral data and Cauchy plot method to obtain the Hamaker constant and showed principal relaxations occur outside the range accessible by analytical spectroscopy. Hamaker constant used in this study are higher than those previously used. Overall, the geometric factor is considerably smaller than is estimated using idealized shapes such as spheres and its dependence on protein size is even weaker. This showed idealized models may severely overestimate the magnitude of dispersion forces; pertinent to solubility and adsorption behavior. The colloidal approach used in this study provides a useful approach to capturing the same types of structural details as atomistic models but in a more efficient fashion.<sup>9</sup> This universal approach to molecular interactions became important when looking at properties of a solution as a whole.

Roth went on to add a note about his improved parameters for Lifshitz Theory calculations. Modified parameters were set to describe the UV dielectric behavior of water. The new parameters fit the original reflectivity data much more closely than the set usually used and lead to significant changes in calculations of Hamaker constants.<sup>10</sup> These new values were used in his previously published study described above<sup>9</sup>. Roth admits there may be a considerable degree of uncertainty in Hamaker constants and explains Cauchy plots are simplifications and should only be used as rough estimates.<sup>10</sup> Because Hamaker constants are only estimations, it would be helpful to develop new methods for detection and/or new equations that do not involve this constant.

Models are becoming more and more complex with time and knowledge. McGuffee and Elcock developed a Brownian dynamic simulation method that modeled over 1000 proteins, all treated with atomic detail.<sup>11</sup> Previous models considered many fewer molecules or looked at the molecule as a

whole, with only group properties. Intermolecular forces were described in this model using an energy function that incorporates electrostatic and hydrophobic interactions and was calibrated to reproduce experimental thermodynamic information. Simulations were performed over a wide range of pH and ionic strengths. The model reproduced trends in experimental second virial coefficient (a method of describing viscosity<sup>1</sup>;  $B_{22}$ ) and translational diffusion coefficient. The model correctly captured changes in  $B_{22}$  values due to a single amino acid substitution, and revealed a new explanation for the difficulties reported previously in the literature in reproducing  $B_{22}$  values for protein solutions of low ionic strength. A strong correlation was found between a residue's probability of being involved in protein-protein contact in the simulations and its probability of being involved in an experimental crystal contact. The simulation model also gave a description of behavior of proteins at very high protein concentrations, suggesting a computational framework for modeling complex cellular conditions and behaviors.<sup>11</sup> If a computational model could be developed, progress in development of high protein concentration solutions could be made much more quickly. This would greatly improve drug development methods, as clinical testing alone takes many years before products can reach the public market. Accelerating any step toward successful drug development give the potential of saving more lives.

As research on protein interactions at high concentrations progresses, more is known that can be applied to successful development of SC drug delivery systems. SC drug delivery eases treatment and allows patients to treat themselves using premeasured syringes. With easier and more convenient medications, patients are more likely to stick with a treatment program, leading to fast and better recovery and/or treatment. SC delivery would also decrease the number of patients housed in hospital for IVs, increasing the care provider: patient ratio, increasing the quality of care received by all while in the hospital. Though widely available commercial SC products may be many years away, investigations being done now and in the past will only speed the process along.

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