Protein-Protein Interaction Measurement: Balancing Complete Characterization with High-Throughput Capability in Formulation Development

Introduction

High throughput screening in the world of pharmaceutical development has dramatically increased the number of approved drugs in the last 20 years, and drug developers increasingly use high throughput screening methods as a basis for lead evaluation. This is true in the area of formulation development for biologics, as well, and there are two metrics, Kd and B, that are competing for acceptance as the best predictor of formulation success in terms of stability, aggregation, and viscosity. This paper will look at some recent literature focused on measuring Kd (Diffusion Interaction Coefficient) in a high throughput manner to predict formulation success. The acknowledged problems revealed in the discussion of each papers' results yield valuable insights into the complexity involved in characterizing protein-protein interactions.

It is well established that a dilute solution thermodynamic parameter, the osmotic second virial coefficient (B value), has quantitative and predictive properties regarding both protein crystallization and protein solubility behavior. For example, to increase the probability of successful crystallization of protein, the crystallization solution should have a B value in the range of approximately -8.0 to -0.2 mol mL/g2 which is referred to as the "crystallization slot" in the literature (Acta Crystallogr D Biol Crystallogr. 1994 Jul 1;50(Pt 4):361-5). As the B value of the crystallizing solution becomes more negative, protein-protein interactions become stronger, often to the point that a shower of crystals, or even amorphous precipitates, form. For more positive B values, protein-protein repulsion increases so that the formation of crystal nuclei, or amorphous aggregates, is more difficult (i.e. the solubility of the protein increases). It is exactly this condition – more positive B values – that is beneficial in protein formulations where solubility and stability are of paramount importance.

It is important to realize that B behavior mimics solubility behavior but does not predict absolute solubility. For example, if it is desired to know how pH affects the solubility of a certain protein, then measuring B value as a function of pH will give results which mimic the solubility behavior as a function of pH. This will be true for any solution variable such as temperature or solution additives (excipients). Thus, in general, solution conditions which increase the B value will result in an increase in protein solubility, or vice versa.

Since B value is established as a good predictor of protein solubility behavior, the question arises whether or not an experimental platform can be derived to accurately measure B value in a high throughput (HT) manner appropriate for formulation screening. Although HT is a vaguely defined term in formulation development, it is generally thought to mean minimum protein consumption, minimum time consumption, and minimally hands-on instrument operation per B value measurement.

Traditional methods for B value measurement include membrane osmometry, analytical ultracentrifugation (AUC), and static light scattering (SLS), and the majority of the work proving B value

related to protein crystallization and protein solubility behavior has been performed using SLS. However, none of the above-listed methods meet the general expectations of HT. Recently, two approaches are touted as being capable for use as a screening tool for protein formulations – dynamic light scattering (DLS) and self-interaction chromatography (SIC).

DLS measures scattered intensity fluctuations in a protein solution (as opposed to absolute scattered intensities required in SLS). Since non-absolute measurements are generally easier to determine than absolute measurements, DLS is inherently easier to perform and less fraught with the experimental rigors required of SLS. The experimental protocol for using DLS as a screening tool for protein formulation is to measure the apparent translational diffusion coefficient, Dapp, as a function of protein concentration in a defined solution condition. The data is then cast as:

$Dapp = D°(1 + kDc)$

D° = infinite dilute value for the translational diffusion coefficient

c = protein concentration (mg/mL)

kD = diffusion / interaction virial coefficient (mL/g)

The value of kD is determined from the slope of a plot of Dapp vs. concentration, where D° is the extrapolated intercept and the slope is D°kD. The interaction coefficient, kD, is comprised of two contributions – a thermodynamic contribution via B value, and a hydrodynamic contribution via the frictional factor, kf, for the protein. The frictional factor is dependent on protein size and shape, and does itself have concentration dependence. In certain solution conditions, if the thermodynamic contribution to kD dominates, then clearly the kD value will closely reflect the B value and trends in kD will mimic trends in B value. ***However, for most solution conditions, there is no apriori way of knowing the relative contributions to kD of the thermodynamic and hydrodynamic terms***. ***Thus, to extract B value from a kD measurement becomes ambiguous unless the hydrodynamic contribution is known.***

Measurement of B values, the sum of all interactive forces at all distances and orientations, can be written in terms of the diffusion coefficient (Kd), the friction coefficient (Kr) and volume (V):

$B = (Kd + Kr + v)/2M$

It is apparent in this form of the equation that Kd only represents a portion of the interactive forces in a protein formulation.

Recent Literature

Summarized below are some of the pertinent findings from recent publications.

1. Weak Interactions Govern Viscosity of Concentrated Antibody Solutions.
Connolly, et al., Biophysical Journal. (2012) 103, 69-78.

The authors investigated 29 different mAbs and kD values were determined by using 60 uL aliquots of protein solution in a plate reader at 1, 5, 10, 15 and 20 mg/mL, also using the relationship: Dapp = D°(1 + kDc)

In addition, sedimentation velocity experiments were performed at similar protein concentrations and the sedimentation interaction parameter, ks, was determined from: 1/Sapp = 1/S°(1 + ksc)

S° = infinite dilution value of the sedimentation coefficient

The working equation to estimate B value was: B = (kS + kD +v) / 2M

From the estimated values of B, an empirical relation between kD and B was obtained for the 29 mAbs: KD = 1.33BM – 8.2

The authors state that this equation is “not universal but limited to molecular types with similar shapes.” This method for determining B, although complete due to the direct measurement of both ks and kd, uses large quantities of protein and is not amenable to high-throughput derivation of B

2. The Role of Electrostatics in Protein-Protein Interactions of a mAb.
Roberts, et al., Molecular Pharmaceutics (2014) 11, 2475-2489.

The authors sought to compare B measurements done by SLS to kD measurements performed using a DLS plate reader. It was noted that “the main motivation for measuring B is that is provides a direct link to protein-protein interaction, whereas the link to kD is less well established, especially for particles with non-spherical shapes.” This is due to the fact that the friction factors are ill defined. In the context of protein-protein interactions of a formulation the kD captures only a partial component of the forces involved. The finding by Roberts et al highlights the incompleteness of kD when identifying protein-protein interactions of a given formulation.

3. Temperature-Ramped Studies on the Aggregation, Unfolding, and Interaction of a Therapeutic Monoclonal Antibody.
Menzen and Friess, Journal of Pharmaceutical Sciences 103:445–455, 2014

In this 2013 investigation of temperature dependent antibody behavior, Menzen et al develop an empirical relationship between B and kd, similar in purpose to the relationship developed by Connolly, et al. However, the relationships differ with the Menzen paper identifying the following relationship (R2 = 0.85): kd = 1.19BM – 6.29

In addition to identifying a significantly different relationship between B and kd the authors find the relationship did not hold for the Fab fragment. And state this, "might be a hint that the TIM *transformation of interaction parameters of MAbs] equation is valid only for the full MAbs and cannot be transferred to individual Fab fragments."

4. Prediction of Colloidal Stability of High Concentration Protein Formulations.
Garidel, et al., Pharmaceutical Development and Technology. (2014) e-pub

The authors measured kD at 1 to 5 mg/mL to predict colloidal stability at 200 mg/mL. Accelerated colloidal stress was imposed by stirring at 5000rpm for 1-3 hours, but results were not conclusive. In contrast to the Connolly paper mentioned above, this paper evaluates kd as a substitute rather than component (with ks) of B. Kd as a substitute for B value is only appropriate under the limited circumstance where sedimentation interaction does not significantly contribute to B value. It was noted that "whereas it is possible to differentiate between net attractive and net repulsive forces by the sign for B, this is not possible for kD." Results showed that opalescence was as good a predictor as kD and opalescence measurements were used to rank the high concentration solutions (220 mg/mL).

5. Viscosity of High Concentrations of Monoclonal Antibodies
Neergaard, et al., European Journal of Pharmaceutical Sciences 49 (2013) 400–410.

The authors used DLS via a plate reader with 35 uL per well at concentrations of 12 mg/mL and below to estimate kD. There was no quantitative comparison between kd and established measurements of protein-protein interactions. This paper concluded with a suggestion that "the measurement of relative radius we presented as a tool to determine PPI at both low and high concentration may serve as a useful screening tool in high concentration formulation development." While apparent hydrodynamic radius (rh), and kd are relatively easy to measure it would take exceptionally strong quantitative evidence to substitute a thermodynamic parameters for a simple secondary measurement such as rh.

6. Method Qualifications and Application of Diffusion Interaction Parameter and Virial Coefficient.
Shi, et al., Int. J. Biol. Macromol. (2013) 62, 487-93.

In this paper, kD was investigated as a B value surrogate for mAbs. The working relation used was
KD = 2MB-kf-v
M = molecular weight of protein (g/mol)
V = partial specific volume of the protein (mL/g)
KD, B, and kf previously defined

The authors used a 96 well plate reader to measure kD using 100uL/sample and protein concentrations up to 25mg/mL. When performing DLS measurements in quadruplicate in a 96-well plate reader format, Shi notes that "the compounded (D & cp) variability of Kd measurements (by a DLS plate reader) may compromise its ability in determining close protein-protein interactions…." Compared to B measurements made via SIC, there is a single experimentally derived factor, the chromatographic retention factor K', which results in less ambiguity. In Shi's study, it was determined that "plate based (Kd) measurement failed to unambiguously determine equivalence or difference from 3 different batches of mAbs expected to have very similar Kd values,…"

Additionally Shi, et al suggests, "To differentiate close interactions, alternative approaches may be pursued such as cuvette-based KD measurement which could effectively reduce location variability from the diffusion coefficient measurement since a single cuvette would be used for all concentrations. Other orthogonal or complementary methods may also be considered…" While cuvettes can provide increased accuracy, the approach would negate the high-throughput nature of the plate based measurements. Orthogonal methods, such as sedimentation velocity studies, can give a complete thermodynamic and hydrodynamic assessment of B value, but at the cost of additional protein and time.

SOLUTION

The diffusion coefficient, Kd, provides a partial indication of protein-protein interaction. Unfortunately, it does not account for the complete set of forces involved in protein-protein interactions. In limited situations where protein-protein interactions are dominated by diffusion components, Kd could serve as a useful proxy for B. Connolly notes, "the empirical relationship between kD and B2 is not universal but limited to molecular types with similar shapes (e.g., IgGs). Determination of B2 across molecular types will necessitate an independent determination of both kD and kS…" On the other hand, direct measurements of B, such as SIC and SLS, account for the sum of **all** forces in protein-protein interactions. While Kd can be supplemented by additional AUC information to determine B value, the additional methods required for complete measurement of protein-protein interactions negatively impacts the high-throughput nature of the method. With the introduction of high-throughput methods for the determination of B value, such as the HSC Technology, it is now possible to accurately measure all of the variables that contribute to protein-protein interactions using a low concentration and volume of protein with a single measurement in a short period of time.