Formulation Optimization Using the HUNK

Measuring ΔG: The Development Pathway to the Optimal Drug Product

The development of successful biologics requires formulations that extend shelf life while simultaneously achieving the final desired Target Product Profile for the Drug Product. Three physical factors are critical in formulation optimization: stability, aggregation and viscosity. The ideal mixture of buffers and excipients to formulate a protein is the one that maximizes stability, minimizes aggregation and keeps viscosity sufficiently low to permit viable high concentration formulations.

Important goals in formulation optimization:

- Minimizing aggregation – from either the native protein or the denatured protein
- Optimizing solubility – without overly compromising stability
- Minimizing viscosity – also without overly compromising stability

These important challenges are being addressed in biopharmaceutical laboratories using the HUNK, a fully automated chemical denaturation system, which provides quantitative data. The HUNK is now being applied from early-stage biologics developability assessment through to later-stage formulations screening and formulations optimization for long-term stability.

As illustrated in Figure 1 the goal of formulation optimization is to maximize the amount of protein in the native active conformation while minimizing the amount in the denatured (and partially denatured) conformation. Ideally the optimal formulation will also minimize self-association of the native protein and prevent denatured protein aggregation.

The HUNK assesses changes to conformational stability and aggregation (native protein and denatured protein) that result from changes to the protein construct itself or to its formulation conditions (buffers and excipients). Since the HUNK provides a quantitative measure of protein unfolding stability (ΔG), the impact on protein stability can be measured as formulation steps are taken to improve solubility and/or viscosity.

**Figure 1.** The goal of formulation optimization is to maximize the amount of protein in the native active conformation while minimizing the amount in the denatured conformations. The optimal formulation will also contribute to minimizing native protein self-association and aid in minimizing denatured protein aggregation. The HUNK data provides quantification of each of these processes.
Formulation Optimization Using the HUNK– The Process

Figure 2 illustrates the thermodynamically-directed formulations optimization process using the HUNK data. As will be discussed in more detail below, protein conformational stability is assessed using the thermodynamic parameter ΔG (the Gibbs free energy of protein unfolding). The dependencies of ΔG as a function of both protein concentration and time are used to assess native protein self-association and denatured protein aggregation.

![Thermodynamic Optimization Flowchart](image)

**Step 1. Measure Initial Conditions.** The thermodynamic protein stability optimization process often starts with measuring the protein stability in the original purification. As illustrated in Figure 3 proteins unfold as denaturant concentration increases. The thermodynamic parameters ΔG, C½ and m are calculated from the denaturation curve, and the amount of denatured protein is calculated directly from the ΔG value.

![Denaturation Curve](image)

**Figure 3.** Left panel: With the HUNK the protein unfolds as the denaturant concentration increases.

Center Panel: Typical HUNK experimental curve for an IgG1 mAb. The parameters ΔG, C½ and m are calculated from the denaturation curve.

Right panel: ΔG determines how much of the protein in the formulation is denatured.
Step 2. Maximize Stability. In the later stages of formulations optimization, excipients will often be selected to aid in increasing solubility, reducing aggregation and decreasing viscosity. These critical attributes are frequently achieved at the expense of protein conformational stability. For this reason buffer conditions such as pH and salt content must be adjusted to maximize protein conformational stability as some of this stability will later be spent as excipients are added to the formulation to achieve the overall required target product profile. Figure 4 illustrates the point that formulations, which stabilize a protein, require higher denaturant concentrations to unfold them and formulations that destabilize a protein require lower denaturant concentration to unfold them.

<table>
<thead>
<tr>
<th>$\Delta G$ (kcal/mol)</th>
<th>% Denatured Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>1.3</td>
<td>10</td>
</tr>
<tr>
<td>2.7</td>
<td>1</td>
</tr>
<tr>
<td>4.1</td>
<td>0.1</td>
</tr>
<tr>
<td>5.5</td>
<td>0.01 (100ppm)</td>
</tr>
<tr>
<td>6.8</td>
<td>0.001 (10ppm)</td>
</tr>
<tr>
<td>8.2</td>
<td>0.0001 (1ppm)</td>
</tr>
<tr>
<td>9.6</td>
<td>0.00001 (0.1ppm)</td>
</tr>
</tbody>
</table>

Figure 4. Left panel: As changes are made to the formulation (e.g. pH, salt content, etc.) the denaturation curve shifts and indicates higher or lower stability for each formulations tested. Center panel: Example; an IgG1 mAb is stabilized by the addition of 100 mM NaCl. Right panel: The amount of denatured protein present in each formation is directly determined by $\Delta G$.

- The goal at this stage of the optimization process is to maximize stability before other excipients that are likely to impose a stability penalty are added to the formulation.
- For example, in a 100 mg/mL subcutaneous formulation: if $\Delta G = 4$ kcal/mol, 100 µg will be denatured. If $\Delta G = 8$ kcal/mol, 0.1 µg will be denatured.

At this stage, pH is one of the most important formulation parameters to evaluate. As shown in Figure 5, the $\Delta G$ values obtained from the chemical denaturation curves of an IgG mAb at multiple pH’s show a definitive stability trend.

Figure 5. Left panel: A typical 3-state chemical denaturation curve for a monoclonal antibody. Center panel: Overlaid chemical denaturation curves for the same mAb at PH 4, 5, 6 and 7. Right panel: Stability ($\Delta G$) trend as a function of pH as determined from the HUNK data.
Step 3. Optimize Solubility and Minimize Aggregation. As the formulations optimization progresses, the focus often turns to finding excipients and excipient concentrations that most effectively increase solubility and minimize aggregation. These excipients may be stabilizing or destabilizing to the protein itself. Common examples of excipients that stabilize the protein native conformation include sugars, polyols, some amino acids, amines, and salting out salts. Sucrose and trehalose are the most frequently used sugars and large polyols are in general better stabilizers than smaller polyols. Figure 6 illustrates an example of the effect of a stabilizing excipient (sucrose) on the chemical denaturation curve of an IgG1 mAb.

Figure 6. Left panel shows the chemical denaturation curve for an IgG1 mAb in the absence of sucrose. Right panel shows the stabilizing effect of sucrose. The ΔG values are 8.99, 9.47 and 9.95 for 0, 0.05 and 0.10 g/mL of sucrose, respectively.

Frequently, excipients have a destabilizing effect on proteins but their use is necessitated for other reasons (i.e. aggregation suppression, solubility enhancement or viscosity reduction). Perhaps the most widely used excipient with these characteristics is arginine. Arginine is used as a solubilizing agent and has been shown to be highly effective at suppressing aggregation. Arginine often increases protein solubility and reduces the viscosity of high concentration formulations. Due to its aggregation suppression ability and its safety in humans, the application of arginine in biologics is rapidly growing. Figure 7 illustrates the effect of arginine on the chemical denaturation curves of two different IgG1 mAbs.

Figure 7. Left panel shows the destabilizing effect of arginine on a mAb as determined by the 2-state chemical denaturation unfolding. Right panel illustrates the destabilizing effect of arginine on a mAb as determined by the 3-state chemical denaturation unfolding.
As illustrated in Figures 6 and 7, changes in protein conformational stability due to changes in formulation are easily determined by measuring changes in ΔG. However, far more information about native protein self-association and denatured protein aggregation is available by also measuring changes in ΔG as a function of protein concentration. As illustrated in Figure 8, in the absence of aggregation, the equilibrium of monomeric protein between the native and denatured states is independent of the protein concentration. However, in the presence of native protein self-association or denatured protein aggregation, the equilibrium process is no longer strictly monomeric and there will be a dependence of the equilibrium (and hence ΔG) on the protein concentration. As illustrated in Figure 9, in the presence of native protein self-association, the native state is stabilized. The degree of native protein self-association is concentration dependent and hence the apparent ΔG will be concentration dependent. In this case the apparent ΔG increases as the protein concentration is increased. Similarly in the case of denatured protein aggregation, the denatured state is stabilized, and the degree of denatured protein aggregation is also concentration dependent and hence the apparent ΔG will again be concentration dependent. In this case, the apparent ΔG decreases as the protein concentration is increased.

**Figure 8.** For monomeric protein, ΔG must be independent of concentration.

**Figure 9.** If ΔG increases with protein concentration, there is aggregation in the native state. If ΔG decreases with protein concentration, there is aggregation from the denatured state.

The goal of formulations optimization is to develop a formulation that optimizes long-term stability while simultaneously meeting the other target product profile objectives such as solubility and viscosity. Meeting these objectives often requires the careful balancing of protein conformational stability and native or denatured protein aggregation. The balancing of conformational stability versus native protein self-association is illustrated in Figure 10.
**Figure 10.** Although addition of NaCl to the formulation improved the protein conformational stability (left panel), elimination of NaCl from the formulation decreased native protein self-association by 15-fold (right panel).

**Step 4. Lower Viscosity.** As with many excipients that increase solubility and reduce aggregation, excipients that decrease viscosity can also negatively impact conformational stability. This may or may not be problematic to long-term stability depending on the starting magnitude of conformational stability and the amount of conformational stability loss due to the addition of the viscosity lowering excipients. Figure 11 illustrates this point. Du & Klibanov (Biotechnology and Bioengineering, Vol. 108, No. 3, 2011) reported the significant viscosity lowering effect of the hydrophobic salt, Phenyltrimethylammonium Chloride (PTA-Cl). Figure 11 shows the impact of PTA-Cl on the stability of a monoclonal antibody. Although the viscosity lowering effect was demonstrated to be dramatic, in this particular example, it is unlikely the protein would possess acceptable long-term stability due to severe loss of conformational stability and consequent increase in the amount of denatured protein in the formulation.

**Figure 11.** For this example mAb, the viscosity lowering agent PTA-Cl lowers conformational stability by such a large amount the formulation would be highly unlikely to provide acceptable long-term stability.
Measuring ΔG: The Development Pathway to the Optimal Drug Product

Monitoring changes in ΔG as a function of systematic changes to the formulation and as a function of protein concentration provides a pathway to thermodynamically-directed formulations optimization toward achieving the target product profile. The chemical denaturation technique is uniquely suited to providing measurement access to ΔG for biologics.

ΔG provides guidance for every step of formulations screening and optimization

The HUNK provides:

- Broad applicability to mAbs, fusion proteins, bi-specifics, ADC’s, replacement proteins, etc.
- Stability and aggregation propensity information directly at room temperature
- Measurement of the amount of denatured protein for each formulation
- The stability “opportunity and cost” of formulation changes to decrease aggregation, increase solubility and reduce viscosity
- A high degree of reproducibility and a thermodynamic fingerprint unique to each protein and formulation

Measurement of ΔG has been successfully applied to essentially all aspects of optimizing biologics formulations including buffer selection and optimization of pH, salts and excipients for stability, solubility and viscosity.