



Development and Optimization of Protein Formulations

DSC Application Note

Introduction



Formulation optimization is best performed early in the development of a biopharmaceutical. Since biopharmaceuticals are primarily proteins or peptides, they need to be stabilized in their active, native form until administration. This application note describes

the use of differential scanning calorimetry (DSC) as a method for rapidly determining optimum solution conditions for protein formulation stability. Screening formulations for stability and using optimized formulations for accelerated and real-time stability studies can expedite the development of a drug.

Formulation Development

An early development decision is choosing whether a biopharmaceutical will be supplied as a liquid, or a lyophilized (freeze-dried) powder. In general, liquid formulations are less expensive to manufacture and easier to use, but need to be stored under refrigerated conditions and tend to be less stable. Freeze-dried proteins are more costly to manufacture and require dissolution before administration, but can be stored at room temperature and may provide enhanced stability. Convenience by the end user and cost are also factors.^{1,2} The formulation scientist must determine if the protein can maintain stability in solution for a sufficient period of time, or can only be kept stable in freeze-dried form.

A protein in aqueous solution is in equilibrium between the native (folded) conformation and its denatured (unfolded) conformation. Hydrophobic interactions and hydrogen bonding are the major stabilizing forces, and these forces have to be overcome for a protein to unfold and denature. Conformational entropy weakens stabilizing forces, allowing the biopolymer to unfold.³ Proteins unfold upon heating or when denatur-

ing chemicals (e.g. sodium dodecyl sulfate and guanidinium hydrochloride) are added to the solution. Denatured proteins tend to be more susceptible⁴⁻⁷ to irreversible chemical processes like proteolysis,⁸ oxidation,⁹ and deamidation,¹⁰ which can lead to inactivation. A denatured protein is also more likely to aggregate, and aggregation can likewise lead to loss of stability and breakdown of the protein.¹¹⁻¹⁴

Prior to formulation development, the protein has to be characterized, which may include determination of its molecular weight, amino acid composition, three-dimensional structure, presence of disulfide bonds, glycosylation, requirements of cofactors, inhibitors, solubility, thermodynamic parameters, functionality, isoelectric point, hydrophobicity, and surface area. All of this information is valuable for designing the optimal formulation of the protein. Using "Rational Drug Design," bioengineered proteins can also be constructed for maximum stability and greatest efficacy in the solution of choice.

A protein's liquid formulation should be favorable for maintaining stability and bioactivity of the biopolymer during production, packaging, storage and shipping, until the ultimate delivery to the target site in the patient. Parameters to consider during formulation development include protein concentration, presence of additives (excipients), pH, temperature of storage, container, exposure to light, air and humidity.

Another factor in formulation development involves the drug delivery mechanism. For example, an intravenously delivered biopharmaceutical needs to be dilutable, and if the protein is poorly soluble, it can precipitate in the bloodstream of the patient. Also, if the drug is injected, the composition of the formulation should not cause tissue damage or pain to the patient. Another consideration is the potential adsorption of protein onto the container or device surface (syringe, pump, etc.)

Stability of a protein is typically determined using various analytical methods, including accelerated and

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real time stability studies. Analysis of aggregation/precipitation, oxidation, proteolytic degradation, and/or disulfide bond shuffling is also typically evaluated. Shipping conditions are also tested to ensure the drug can be delivered without loss of bioactivity.

Optimization of the formulation for stability, and testing stability during storage in a variety of formulation candidates is costly and time consuming. A technique that can identify the best formulation candidates will accelerate development. Differential scanning calorimetry (DSC) is a technique for screening formulations for thermostability, and formulations with the best stability may then be used for further drug development.

Differential Scanning Calorimetry and Formulation Development

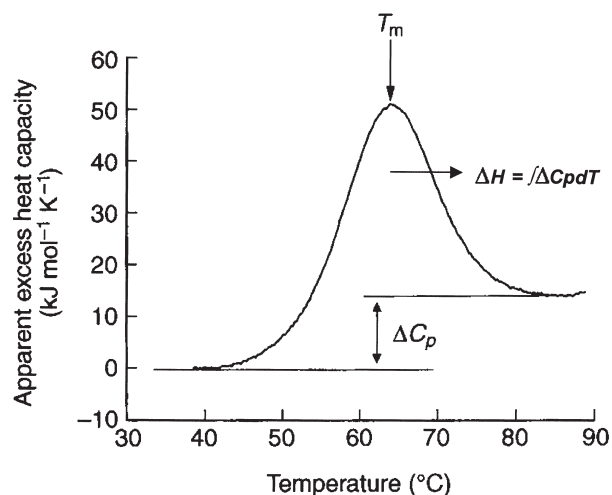
A DSC instrument has a sample cell (containing biomolecule plus buffer) and a reference cell (filled with buffer only); power is supplied to heaters to raise the temperature of the cells at a constant rate. During this temperature increase, the instrument monitors the temperature difference between the sample and reference cells. The difference in heat uptake between the cells required to maintain equal temperatures in both cells, determines the apparent excess heat capacity (Figure 1). The temperature midpoint of the transition for the enthalpy change (T_m) occurs when the protein goes from native to denatured form. At the T_m , 50% of the protein is in the native state, and 50% is in the denatured state, assuming a two-state transition (Figure 1). Some proteins with different regions of activity or more than one structural domain can have more than one T_m . The scientist can focus on one or two T_m s that show the greatest effects due to formulation changes.

T_m is an indicator of thermostability, and in general, the higher the T_m , the more stable the protein. With a higher T_m the protein is less susceptible to unfolding and denaturation at a lower temperature as well. By interrogating various conditions and additives, DSC can determine formulations with the highest T_m that will correspond to the optimal formulation(s) for stability.^{4,5,7,15,16}

During a chemical process heat is either released (exothermic) or absorbed (endothermic). The transition from native to denatured protein is generally endothermic. The change in enthalpy (ΔH) during the conformational transition is measured by integration of the area under the transition (Figure 1). The heat capacity (C_p) of the denatured protein is typically higher than the C_p

of the native protein, resulting in a positive ΔC_p for thermal denaturation (Figure 1).

FIGURE 1



Typical DSC thermogram. This DSC scan was carried out on a dilute protein solution, where the protein undergoes a transition from a compact, native state at low temperature to an unfolded, denatured state at high temperature. The apparent excess heat capacity of the protein was measured, based on the difference in the heat capacity of the protein in buffer, and buffer alone. The T_m , ΔH and ΔC_p of the transition are calculated by fitting the data to a two-states transition model using non-linear least square regression analysis.

The VP-Capillary DSC Platform and VP-DSC are utilized in the study of biopolymers in solution. The VP-Capillary DSC Platform (Figure 2) is specifically designed for T_m screening of multiple formulations using high sample throughput (up to 50 samples in a 24 hour period), fast scan rate (up to 250 °C/hour), and a fully integrated autosampler for hands-off operation. See Table 1 for a comparison of features of the VP-Capillary DSC Platform and VP-DSC.

FIGURE 2



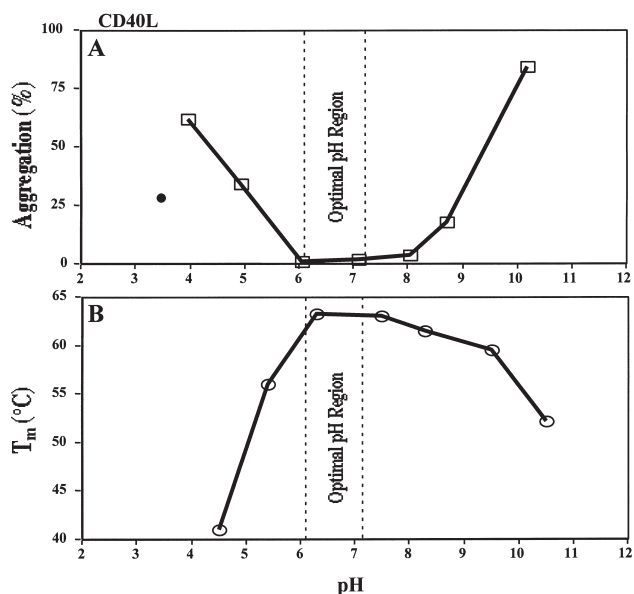
Liquid Formulation Strategies

In formulation development, the main question is: what solution conditions offer the greatest stabilization of native protein? The conditions which result in the highest T_m typically maintain the protein in its native

state for the longest time at lower temperatures as well. Using DSC, different pHs and buffers are screened first, followed by excipients and preservatives.

1. Buffer and pH Optimization. In Figure 3 the T_m of protein CD40L was plotted against the pH. The aggregation of CD40L was also determined, after incubation at 37°C for 7 days. The T_m optimum correlated with the pH conditions where aggregation was minimized.¹⁶ This correlation between pH, T_m , and aggregation was also seen with macrophage colony stimulating factor.⁴

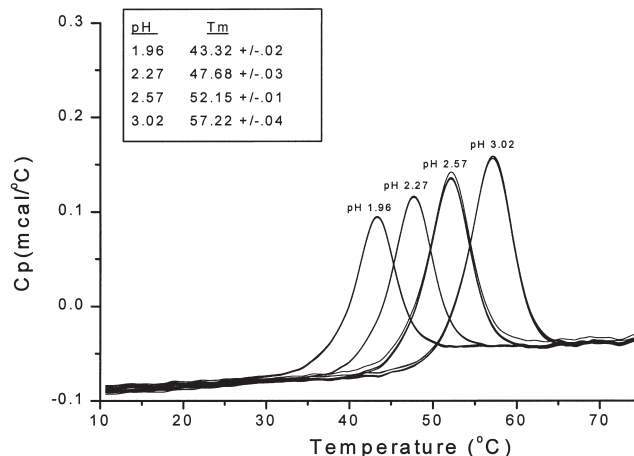
FIGURE 3



Stability behavior of CD40 ligand (CD40L) correlating aggregation response as determined by size exclusion chromatography (SEC) (A), and the T_m determined by DSC (B) as a function of pH. The bracketed area represents the optimal pH range where T_m is maximized and aggregation is minimized. (From Reference 16.)

Measuring T_m changes of proteins by DSC is a relatively simple process. Figure 4 shows T_m changes of chymotrypsinogen when pH is increased, as measured with the VP-Capillary DSC Platform. The T_m increased with increasing pH, indicating greater stability of the native form of chymotrypsinogen at higher pH.

FIGURE 4



T_m shift of chymotrypsinogen with pH. Chymotrypsinogen solutions (pH 1.96, 2.27, 2.57, and 3.02) were prepared and added to the wells of 96-well plate. Five samples were used for each pH. Matched reference buffers were also placed in the 96 well plate. DSC scans were performed with the VP-Capillary DSC Platform. The DSC data shown here are after buffer-buffer reference scan subtraction. The inset has the T_m data for each pH, and standard deviation.

2. Excipients. These are additives that can improve the stability of the protein. They include sugars, amino acids, antioxidants, polymers, alcohols, glycerol, and surfactants.

Once the optimum pH and buffer are determined, different excipients are added to the protein solution. If an excipient increases the T_m , the native form of the protein is more stable with the excipient than without it.

Excipient screening was used during the liquid formulation development of Interleukin-1 Receptor (IL-1R).⁵ There were two major transitions for IL-1R, one with T_m near 48°C and the other near 66°C. (Figure 5). The transition with the T_m at the lower temperature was chosen to screen for excipients. The strategy was to look for excipients that would raise the T_m of the low temperature transition, indicating a positive change in native protein stability. Twenty-three excipients were screened (Table 2).

| | VP-DSC | VP-Capillary DSC |
|---|--------------------------|--|
| Active cell volume | 500 μ l | 130 μ l |
| Volume required to fill | 0.8 – 1.0 ml | 350 μ l in well 275 μ l in injection needle |
| Typical protein concentrations required | 0.02 – 0.1 mg/ml | 0.3-0.5 mg/ml (T_m) >1.5mg/ml (ΔC_p and ΔH) |
| Maximum scan rate | 90°C/hour | 250°C/hour |
| Temperature range | -10° to +130°C | -10° to +130°C |
| Typical time per scan | 60 – 150 min. | 35 – 55 min. (depends on scan rate and temperatures) |
| Maximum scans per day | 4 – 6 (manual) in 8 hrs. | ~ 50 (unattended) in 24 hrs. |
| Automated cell filling and washing | No | Yes |
| Samples per 96 well plate | Not Available | 48 |
| Solid samples | Yes (with accessory) | No |
| PPC capabilities | Yes | No |

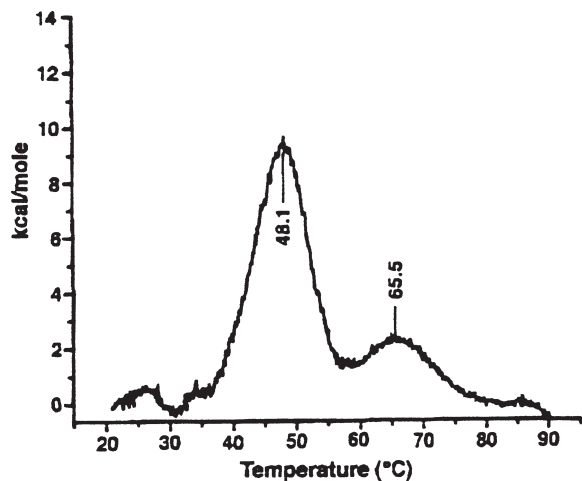
| | Excipient | Concentration (g/ml) in Buffer | Mole Ratio [M_s / M_p]* | T_m (°C) |
|-------------|-------------------|--------------------------------|-----------------------------|------------|
| | Control** | 0.00 | — | 48.1 |
| | Ascorbic Acid | 0.05 | 2037 | 36.7 |
| Sugars | Mannitol | 0.0517 | 2037 | 46.7 |
| | Lactose | 0.0972 | 2137 | 49.7 |
| | Sucrose | 0.0972 | 2037 | 49.7 |
| | Glucose | 0.0512 | 2037 | 49.6 |
| Polymers | PVP (MW 10,000) | 0.01 | 7 | 48.9 |
| | PEG (MW 300) | 0.0003 | 7 | 49.4 |
| | PEG (MW 1000) | 0.001 | 7 | 49.1 |
| | PEG (MW 3350) | 0.00335 | 7 | 48.7 |
| | Dextran 40 | 0.0392 | 7 | 48.0 |
| Polyols | Glycerol | 0.01 | 779 | 48.7 |
| | Ethanol | 0.0051 | 779 | 48.6 |
| | Ethanol | 0.05 | 7617 | 43.8 |
| Salts | NaCl | 0.00584 | 717 | 53.1 |
| | CaCl ₂ | 0.0111 | 717 | 41.1 |
| Amino Acids | Glycine | 0.01 | 955 | 46.2 |
| | L-Lysine | 0.01947 | 955 | 48.3 |
| | L-Cysteine | 0.01614 | 955 | 51.3 |
| | L-Alanine | 0.01187 | 955 | 46.2 |
| | L-Arginine | 0.0232 | 955 | 49.1 |
| Surfactants | Pluronic F68 | 0.0001 | 4 | 46.6 |
| | Tween 80 | 0.001 | 5 | 45.8 |
| Combination | Glucose/NaCl | 0.0512 / 0.00584 | 2037 / 717 | 52.2 |

* M_s = moles of excipient / M_p = moles of protein

**Control buffer is 20 mM citrate buffer, pH 6.0. Excipients added to this buffer.

(From Reference 5.)

FIGURE 5

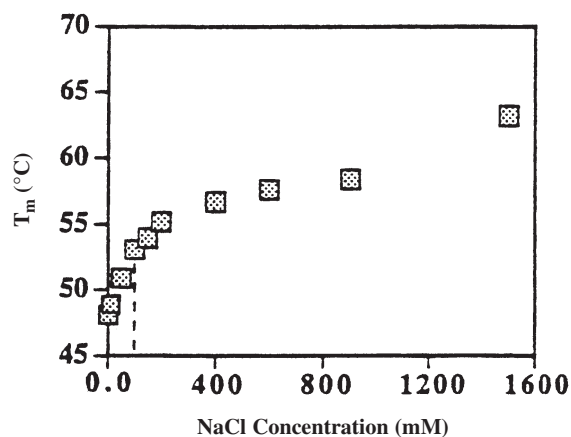


DSC thermogram of IL-1R, 5 mg/ml in 20 mM sodium citrate buffer (pH 6.0) with no excipients. The scan was performed with a MicroCal MC-2 DSC. Two T_m s were observed at 48.1 °C and 65 °C.

(From Reference 5.)

3. Ionic Strength. The ionic strength of the buffer is adjusted to determine if an increase in T_m can be achieved by addition of salt. For IL-1R, the addition of 100 mM NaCl had the greatest stabilizing influence, at modest ionic strength shifting the T_m from 48 to 53°C. This stabilizing effect suggested a direct interaction between salt ions and charged groups of the protein.⁵ The T_m of IL-1R continues to increase with increasing salt, even when NaCl is 1500 mM, well above the concentration needed to saturate all the charged sites (Figure 6). These data suggest that salt ions affect water structure, which also plays a role in protein conformational stability. Both charge-charge interactions, and changes in water structure, add stability to native IL-1R structure.

FIGURE 6



Plot showing T_m of IL-1R with addition of NaCl. The 100 mM concentration is shown by the dashed line.

(From Reference 5.)

4. Preservatives. When a drug is supplied in a multi-dose format, preservatives are added to help prevent microbial growth. However, these preservatives may have a destabilizing effect on the protein. The effect of preservatives on the T_m of IL-1R was also examined.⁵ The preservatives meta-cresol, phenol, and benzyl alcohol destabilized IL-1R, based on the shift of both temperature transitions to lower temperatures (Table 3). The DSC data determined the order of the stability of IL-1R in the three preservatives – phenol produced the highest T_m , followed by meta-cresol and benzyl alcohol. The DSC stability data also correlated with aggregation of IL-1R, as measured by size exclusion chromatography. The higher the T_m , the less aggregation was observed after seven and sixty days.

| TABLE 3. Effects of Preservatives of Interleukin-1 Receptor: Comparison of T_m and size exclusion chromatography | | | | | | | |
|--|---------------|---------------|---------------|-------|----------|-------|----------|
| | | | SEC | | | | |
| DSC | | | 7 Days | | 60 Days | | |
| | T_{m1} (°C) | T_{m2} (°C) | T_{m3} (°C) | Agg % | Native % | Agg % | Native % |
| Control | 50.8 | 53.7 | 66.3 | 0.66 | 98.93 | 1.50 | 97.54 |
| 0.065% Phenol | 50.3 | 53.4 | 66.5 | 1.02 | 98.62 | 3.07 | 96.02 |
| 0.1% m-Cresol | 48.4 | 51. | 65.8 | 1.37 | 98.25 | 5.1 | 93.92 |
| 0.9% Benzyl Alcohol | 45.2 | 48.5 | 63.6 | 2.93 | 96.92 | 16.46 | 83.09 |

Control solution: 20 mM sodium citrate buffer, pH 6.0, 100 mM NaCl. For size exclusion chromatography (SEC), IL-1R solution stored at 37°C for indicated time prior to chromatography. Agg % = % aggregation, determined by integration of high molecular weight protein peak after SEC; native % = % native IL-1R, determined by integration of main protein peak after SEC. (From Reference 5.)

The best formulation candidates, based on T_m screening, are then evaluated by accelerated stability studies. The protein is prepared in different formulations, then stored at 37°C. The amount of aggregation is determined by size exclusion chromatography at intervals during the accelerated stability study, and the protein is analyzed using SDS-PAGE to check for proteolysis.

Finally, real-time stability studies need to be performed with the best formulation candidates to determine the shelf life of the protein. Bioassays and analytical tests are performed throughout the study to ensure the protein is still active and viable.

Summary

DSC data are useful as a predictor of the stability of a protein in solution. The T_m indicates thermostability, and T_m determination in different formulations is an approximate measure of the susceptibility to aggregation and other irreversible changes at lower temperatures. Formulations with the best thermostabilities are chosen for further stability, shelf life, and shipping studies. The use of DSC can save time and money in formulation development.

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