Optim® 1000: Comparison of Optim with Standard Methods to Determine Thermal Unfolding Mid-point, $T_m$

Technical note

The definition of $T_m$

The structural stability of a protein molecule is often defined as its ability to maintain native structure when exposed to various forms of stress. Failure to maintain the correct native structure can lead to loss of function and potentially aggregation which, for therapeutic proteins, can have a range of negative consequences such as reduced production yield, loss of efficacy and increased immunotoxicity.

Therapeutic proteins experience a wide range of stresses during development, production, transport, storage and delivery and it is critical that the protein retains its native structure during these processes.

A widely used measure of the thermal structural stability of a protein is the temperature at which it unfolds from the native, folded state to a denatured, unfolded state. For many proteins this unfolding process occurs over a narrow temperature range and the mid-point of the transition is often termed the ‘melting temperature’ or ‘$T_m$’. This value reflects the thermal structural stability of the protein and is related to the Gibbs free energy of the protein (the energy difference between the native and denatured states). In practice the term ‘$T_m$’ can be used in different ways and so, for the sake of clarity, a short discussion is presented in this technical note.

Formally, $T_m$ can be defined as the temperature at which there is an equal population of folded and unfolded proteins in solution. In order for an experiment to accurately determine an unambiguous $T_m$ a number of criteria must be fulfilled: the protein should have a single cooperative unfolding transition, the transition should be from fully folded to fully unfolded populations, the transition should be reversible, the unfolding and folding transitions should take place in a time period much shorter than the rate at which the temperature is increased during the experiment.

For the large multi-domain proteins that are typically of interest as biopharmaceuticals, these conditions are not often met. As a result, the $T_m$ values measured by various analytical techniques are often ‘apparent’ or ‘observed’ transition temperatures rather than melting temperatures according to the formal two-state definition. For practical purposes, the apparent $T_m$ of a given protein in a given solvent environment is still a valuable and widely used measure of the thermal stability of a protein which is useful when looking at the effects of formulation, for example, on the thermal stability when it is changes in $T_m$ that are of importance. In the remaining discussion we therefore define $T_m$ as the midpoint of an observed temperature-induced unfolding transition.

Measuring $T_m$ with the Optim 1000 using intrinsic protein fluorescence

In order to determine the $T_m$ of a protein, the Optim 1000 applies a temperature ramp to up to 48 samples at a time and monitors the tertiary structure of the proteins by measuring changes in the intrinsic fluorescence spectra.

Most proteins contain one or more aromatic amino acid residues, which due to their hydrophobic nature tend to be buried inside a native, fully folded protein. These aromatic...
residues fluoresce when excited with UV light and the emission is affected by the solvent environment around the residue. When a protein unfolds, either partially or fully, the residues become more exposed to the solvent environment and the fluorescence emission changes. Figure 1(a) shows examples of the fluorescence spectra of native and unfolded protein and figure 1(b) shows some data recorded on the **Optim** 1000 that has undergone primary analysis to plot peak position as a function of temperature followed by secondary analysis to yield the T$_m$ of the transition under conditions of increasing sorbitol concentration. The protein evolves from a fully folded configuration with a peak position of ~330 nm to an unfolded configuration with a peak position of ~339 nm. It can be seen in this particular example that adding sorbitol increases the T$_m$ of the protein and is therefore acting to increase the protein’s thermal structural stability.

![Figure 1](image)

**Figure 1.** Example use of intrinsic protein fluorescence in the **Optim** 1000 to monitor an unfolding transition for an IgG. (a) Intrinsic fluorescence spectrum of native and unfolded IgG – note the change in intensity and shift in peak position. (b) Effect of adding sorbitol on the thermal unfolding transition temperature. Increasing the amount of sorbitol increases the T$_m$ (red lines).

**Comparison of Optim with other T$_m$ determination techniques**

The use of intrinsic fluorescence has been selected for use in the **Optim** 1000 as the optimum method to monitor thermal unfolding of proteins in biopharmaceutical development applications due to its speed, sensitivity and compatibility with very small sample volumes and a very wide range of buffers and solvent conditions. Alternative methods to measure the T$_m$ of proteins in solution with examples are discussed below.

**Differential scanning calorimetry (DSC):** In DSC the protein sample in solution is placed in a metal cell and gradually heated up whilst the amount of energy input required to increase the temperature of the sample by 1 °C is measured. When the protein in solution unfolds, more energy is required to increase the temperature of the sample (i.e., there is an apparent increase in the heat capacity of the sample). Plotting energy flow as a function of temperature reveals a peak – the centre of which is generally taken to be the T$_m$. DSC is widely used to determine protein T$_m$ and can, if performed carefully, give accurate and reproducible results. Care is still needed in defining a T$_m$ value for non-ideal proteins.

Figure 2(a) shows a typical DSC trace for a protein in two different pH buffers. If the protein has multiple domains unfolding at different temperatures then it can be difficult to assign an accurate, single T$_m$ to the protein. Similarly, if the protein aggregates once it has unfolded (which is very common with IgGs, for example) a large exothermic feature is observed in the DSC trace that can significantly distort the endothermic unfolding peak, again making accurate
determination of $T_m$ difficult. Figure 2(b) shows a DSC trace for an IgG sample and demonstrates multiple domains unfolding at different temperatures and rapid aggregation on unfolding.

Figure 2. Example DSC data. (a) DSC curves for cytochrome C at pH 3 (black) and pH 4 (red). This protein has a single domain which undergoes a highly co-operative unfolding transition and does not aggregate on unfolding, resulting in well-resolved exothermic peaks from which the $T_m$ can be determined. (b) DSC plot from an IgG sample. This protein has multiple domains which unfold at different temperatures and undergoes rapid aggregation on unfolding – the combination of which makes an accurate determination of $T_m$ challenging with DSC.

Figure 3. Comparison of Optim 1000 generated unfolding curves (blue) with DSC traces (black) for Cytochrome C at pH 3 and pH 4.

The advantages that the Optim 1000 has over DSC are vastly reduced sample volume consumption and a huge increase in speed. Commercially available DSC machines can only apply a temperature ramp to one sample at a time, whereas the Optim 1000 can heat and monitor 48 samples at a time. The minimum sample volume and concentration for a DSC measurement is currently 30 µL at 0.1 mg/mL (3 µg of protein per measurement), compared to the 0.1 µg required by the Optim 1000.

Figure 3 overlays Optim thermal unfolding data on corresponding DSC data for cytochrome C in two buffers at different pH levels. The use of fluorescence as a tertiary structure probe in the Optim 1000 has the added advantage that more subtle changes in tertiary structure, for example as a result of changing pH, may be measured and chemical unfolding data...
generated, neither of which are readily possible with DSC. It is important to note that fluorescence and DSC monitor different aspects of protein structure and as such may in some cases give slightly different values for $T_m$ and complementary information.

**Circular Dichroism (CD):** CD spectroscopy uses the differential absorption of left and right circularly polarised light in the UV region of the spectrum to monitor the secondary and tertiary structures of proteins in solution. Equipped with a suitable temperature cell, a CD instrument may be used to perform temperature ramps and make measurements of the protein melting temperature. Compared to fluorescence spectroscopy, CD has the advantage that it is possible to monitor both secondary and tertiary structure (although usually not at the same time). However, monitoring only the tertiary structure is generally all that is required to record a protein unfolding curve or look for smaller solvent environment-induced changes in the higher order structure. Currently there are no CD instruments capable of simultaneously heating multiple samples and recording spectra, whereas the Optim 1000 can run 48 samples at a time. The sample consumption for CD tends to be significantly higher than the Optim 1000. It is also worth noting that the far UV (secondary structure) part of the CD spectrum can be adversely affected by absorption by some common buffers and formulation components and the whole CD spectrum can be significantly affected by aggregation of the sample on unfolding.

**Example applications of $T_m$ determination with Optim**
The ability of the Optim 1000 to rapidly obtain high quality thermal unfolding data from up to 48 samples in a single experiment whilst consuming very small quantities of protein means that it is especially well suited to biopharmaceutical pre-formulation and formulation development.

**Pre-formulation development:** The Optim 1000 may be used to screen many candidate molecules and identify those with the highest thermal structural stability or to investigate the effects of typical processing environments on protein structural stability. The information provided can then be used to help select structurally stable candidate molecules that are amenable to manufacture. The information gained may also be used as part of a ‘Quality by Design’ (QbD) approach to biopharmaceutical development where greater emphasis is placed on gathering relevant information about a candidate as early as possible so that this may then be used to rationally select candidate molecules and develop suitable processes.

**Formulation development:** The small sample requirement and rapid measurements mean that the Optim 1000 can map more of the potential formulation ‘design space’ giving the best chance of selecting the optimum formulation. Optim’s unique features also offer the potential to help move the formulation process to earlier in the development pipeline thus reducing risks of problems occurring later and hopefully accelerating subsequent progress. These benefits are particularly beneficial if a QbD approach is being taken to product development.

**Summary and Conclusions**
The thermal transition temperatures ($T_m$) of up to 48 protein samples in solution can be measured in the Optim 1000 with as little as 0.1 µg of protein per sample. This is at least fifty times faster and consumes significantly less protein than alternative technologies to obtain the same information.

The Optim 1000 uses intrinsic protein fluorescence as a means of monitoring tertiary structure and to determine at what temperature a protein unfolds. This method has been selected as being optimum for obtaining rapid, high-quality protein unfolding data from multiple, very small-volume samples. The quality of the data compares with that obtained using alternative techniques but consumes much less sample and takes much less time.