Biophysical characterization approaches to aid the selection of protein formulations by predicting their physical stability during long-term storage

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Biophysical characterization approaches to aid the selection of protein formulations by predicting their physical stability during long-term storage

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July 2019
Protein stability aspects

**Conformational stability**

\[ K_{eq} \]

Folded protein ↔ Unfolded protein

**Colloidal stability**

Weak interactions

**Interfacial stability**

**Chemical stability**

R-CH-COOH + H₂O \[ \rightarrow \] R-COOH + NH₃

Protein aggregation due to poor physical stability

Native folded protein → Partially unfolded protein → Completely unfolded protein

- Reduced biological activity
- Immunogenicity concerns
- Failure to meet regulatory expectations

Aqueous phase

Nuclei

Aggregates
How to select formulations where protein aggregation is suppressed during storage?

- **Time**
- **Effort**
- **Costs**

- **Stability-indicating methods**
- **Accelerated stability studies**
- **Long-term stability studies**

Our aim is to test and improve the predictions from stability-indicating methods.
Thermal denaturation methods

- DSC
- nanoDSF
- ICD
- ReFOLD

Isothermal methods
Differential Scanning Calorimetry

- DSC
- ICD
- nanoDSF
- ReFOLD
Differential scanning microcalorimetry can be used to determine the (apparent) protein melting temperature $T_m$ in different formulations.

Basic assumption of this approach:
The long-term stability of the formulations correlates with the (apparent) protein melting temperatures.
Correlation between prediction from melting temperature of GCSF and storage stability at 4 °C (Youssef and Winter 2013)

Best 50 % prediction

Best 20 % prediction

Storage stability ranking

Outliners

Other examples showing that DSC can provide only some predictions for storage stability:

Interleukin-1 Receptor (IL-1R) Liquid Formulation Development Using Differential Scanning Calorimetry

Richard L. Remmele, Jr.,1,3 Nancy S. Nightlinger,1 Subhashini Srinivasan,2 and Wayne R. Gombotz1

Examination of Thermal Unfolding and Aggregation Profiles of a Series of Developable Therapeutic Monoclonal Antibodies

Mark L. Brader,6 Tia Estey, Shujun Bai,7 Roy W. Abston, Karin K. Lucas,7 Steven Lantu, Pavel Landsman, and Kevin M. Maloney
The $T_m$ approach with DSC in brief

Measuring $T_m$ with differential scanning calorimetry (DSC) offers:
- **Some predictions** which formulations are stable during storage

However, this approach had the following limitations:
- **Low-throughput**: only one sample per run possible (ca. 3 samples per day)
- **Sample heating**: some excipients change properties with temperature
- **Large sample volume**: 550 µl measurement volume -> 800 µl handling volume
- **Limited prediction reliability**: “$T_m$ showed a limitation in predicting the exact ranking”
DSC

Nano „Differential Scanning Fluorimetry“

ICD

nanoDSF

ReFOLD
nanoDSF measures protein unfolding by detecting changes in the intrinsic protein fluorescence.

nanoDSF provides melting temperatures like other methods faster and with less sample.

<table>
<thead>
<tr>
<th>µDSC</th>
<th>near-UV CD</th>
<th>nanoDSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>≈ 550 µL</td>
<td>≈ 600 µL</td>
<td>≈ 10 µL</td>
</tr>
<tr>
<td>≈ 3 samples/day</td>
<td>≈ 6 samples/1.5 h</td>
<td>≈ 48 samples/1.5 h</td>
</tr>
<tr>
<td>Extensive hands-on time</td>
<td>Medium hands-on time</td>
<td>Very short hands-on time</td>
</tr>
</tbody>
</table>
Rapid sample-saving biophysical characterisation and long-term storage stability of liquid interferon alpha2a formulations: Is there a correlation?

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we investigate the effect of pH and ionic strength on protein stability. The predictions from the sample-saving biophysical characterisation are validated by long-term stability studies at 4 °C and 25 °C for 12 months on selected formulations. Interferon alpha2a shows minimal aggregation in 10 mM sodium acetate buffer with pH 4 and low ionic strength. The latter is indicated by the rapid sample-saving biophysical characterisation and confirmed by the long-term stability data.
The $T_m$ approach with nanoDSF in brief

Measuring $T_m$ with nanoDSF offers:

- **Some predictions** which formulations are stable during storage
- **High-throughput**: typically 48 samples in 1.5 hours
- **Small sample volume**: 10 µL for one measurement

However, this approach has the following limitations:

- **Sample heating**: some excipients change properties with temperature
- **Limited prediction reliability**: $T_m$ shows a limitation in predicting the exact ranking
Thermal denaturation techniques like DSC and nanoDSF work by increasing sample temperature to measure melting temperatures.

Overall, as a formulation development strategy, it is useful to increase $T_m$ in the initial stages for poorly stable proteins, at least up until the mechanistic limit is reached whereby $f_{T>37^\circ C} < 10^{-3}$. For degradation of GCSF at 37 °C, this limit was reached at approximately $T_m > 55$ °C, where $\ln v_{mon} < 4$ (% day$^{-1}$). Beyond that point, a very different formulation strategy would be required. Protein engineering to remove aggregation hotspots, minimize local unfolding dynamics, increase the net charge, or remove hydrophobic surface patches could be considered.

High throughput thermal denaturation is a valuable technique to determine the melting temperatures of therapeutic protein candidates in early stage development when the amount of material is limited. When it comes to formulation studies, thermal denaturation techniques in general are (alongside other pitfalls discussed in the introduction) limited by the fact that the increase in temperature can change key properties of the excipients (i.e. pH of the buffer system). Care should be taken when such measurements are conducted. pH screenings based on $T_m$ values should be performed only in buffers with $\Delta$pH/$\Delta$T close to zero. After the pH range of maximum thermal stability of a protein is
Isothermal Chemical Denaturation (ICD) for protein formulation

Increasing concentrations of a chemical denaturant are used to cause protein unfolding

The ICD data can provide the following protein stability-indicating parameters:

- $C_m$ – the “melting” concentration of denaturant needed to unfold 50% of the protein;
- $m_G$ – the “m-value” is an indicator for the cooperativity of the unfolding;
- $dG$ – the Gibbs free energy of protein unfolding;

The true dG allows to calculate the fraction of unfolded protein species.

\[ dG = -RT \ln(K_{eq}) \]

<table>
<thead>
<tr>
<th>dG (kcal/mol)</th>
<th>% Unfolded protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50</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</td>
</tr>
<tr>
<td>6.8</td>
<td>0.001</td>
</tr>
<tr>
<td>8.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>9.6</td>
<td>0.00001</td>
</tr>
</tbody>
</table>

dG is a measure of the protein conformational stability at a given temperature.

Determining the true dG is possible only when the unfolding of the protein in the denaturant is fully reversible.
The concentration dependence of the apparent $dG$ can indicate whether a protein is likely to aggregate from its native or unfolded state.

Concentration dependence of the Gibbs free energy (dG) of protein unfolding

This approach is useful and provides orthogonal information during the selection of stable protein formulations

Protein aggregation at 40 °C

The $dG$ and $dG_{\text{trend}}$ approach with ICD in brief

Measuring $dG$ and the concentration dependence of $dG$ with ICD offers:

- **Some predictions** which formulations are stable during storage
- **No sample heating**

However, this approach had the following limitations:

- **Medium throughput**: Depends on the experimental set-up
- **Medium to high sample volume**: Depends on the experimental set-up
- **Limited prediction accuracy**: The exact order of the formulations is still not known
Isothermal Refolding Assay to Assess Protein Aggregation
Basic assumption of this approach:
The aggregation of the partially unfolded protein during refolding correlates with the long-term storage stability.

Protein aggregation during dialysis refolding depends on the formulation

SEC-MALS chromatograms of native and refolded mAb-A

Effect of formulation on the relative monomer yield of mAb-A after refolding from urea

Can this approach rank the storage stability of the formulations?

Svilenov, H. and Winter, G., 2019.. *Eur J Pharm Biopharm*, 137, pp. 131-139
The relative monomer yield from the ReFOLD assay correlates well with the amount of protein aggregates detected after long-term storage for 12 months at 4 °C.

Formulations of mAb-A

Formulations of mAb-B

The relative monomer yield from the ReFOLD assay correlates well with the number of subvisible particles detected after storage for 12 months at 25 °C.
The ReFOLD assay offers:

- **Good predictions** which formulations are stable during storage
- **No sample heating**
- **Medium to high throughput**: 96 well plate, <48 hours to complete
- **Small to medium sample volume**: between 10 and 100 µL
Which stability-indicating method to choose?

- Thermal denaturation methods
  - DSC
  - nanoDSF

- Isothermal methods
  - ICD
  - ReFOLD
How different methods can be combined in formulation development

New therapeutic protein candidate

- ≥ 50 formulations
- ≥ 20 formulations
- ≈ 5 formulations
- ≈ 1-2 formulations

Thermal denaturation

Isothermal characterization

Accelerated stability studies

Long-term stability studies

nanoDSF (DSC as backup)
Low volume, Fast, Useful, Limitations

ReFOLD (also ICD, DLS, SLS)
Low volume, Better predictions

Storage at 25 °C

Storage at 4 °C

Protein solution with optimized physical stability
In summary:

- Use a combination of stability-indicating methods
- Know the strengths and limitations of each method
- Validate novel predictive methods with long-term stability data
Thank you for your attention!