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Advance microcalorimetry beyond Kd and Tm

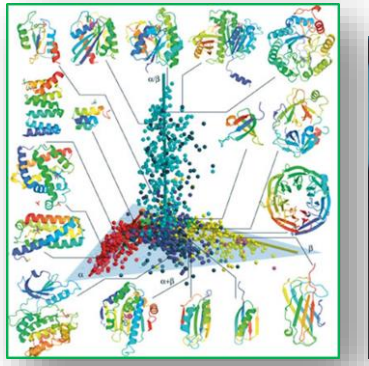
Natalia Markova, PhD

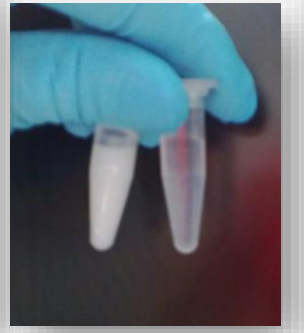
ANALYTICAL STRATEGIES FOR NOVEL THERAPEUTIC MODALITIES
Virtual Workshop - 22nd November 2021

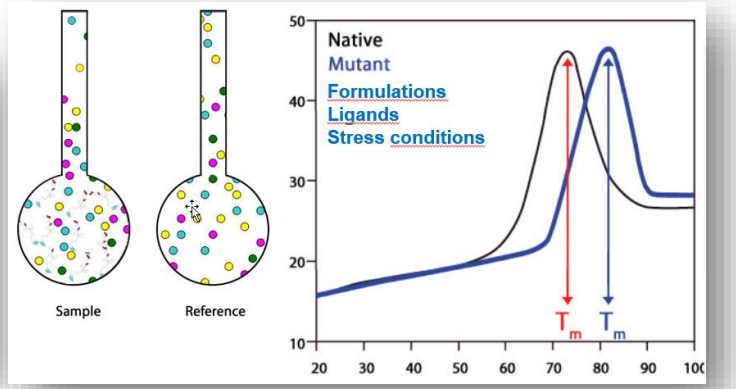
Differential Scanning Calorimetry, DSC offers universal thermal stability assay

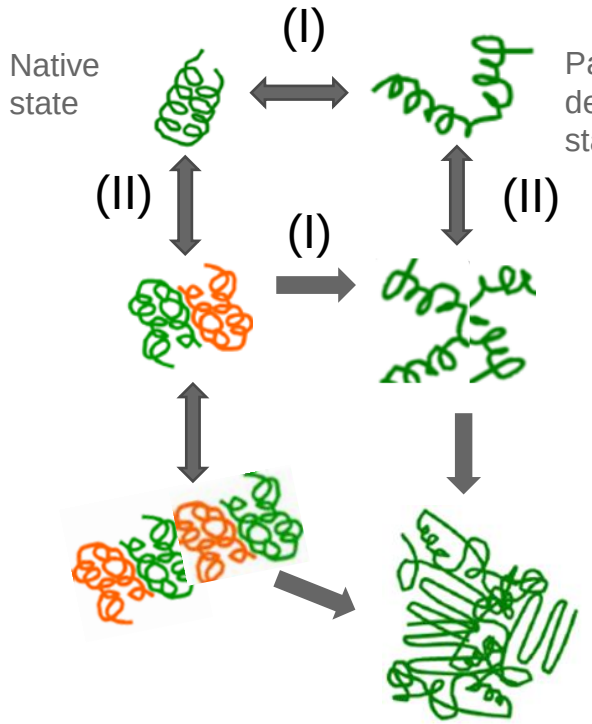
DSC reports on conformational and colloidal instability
and can inform on chemical instability too!

- ✓ Heat as universal readout directly related to protein unfolding event
- ✓ No optical artifacts, works in turbid solutions
- ✓ Work in most of buffers and in biofluids
- ✓ Study protein samples as they are
- ✓ Minimum assay development
- ✓ Inform on stabilization of individual domains
- ✓ Deliver multiple descriptors of protein stability
- ✓ Fingerprint thermal stability for biosimilarity and comparability studies









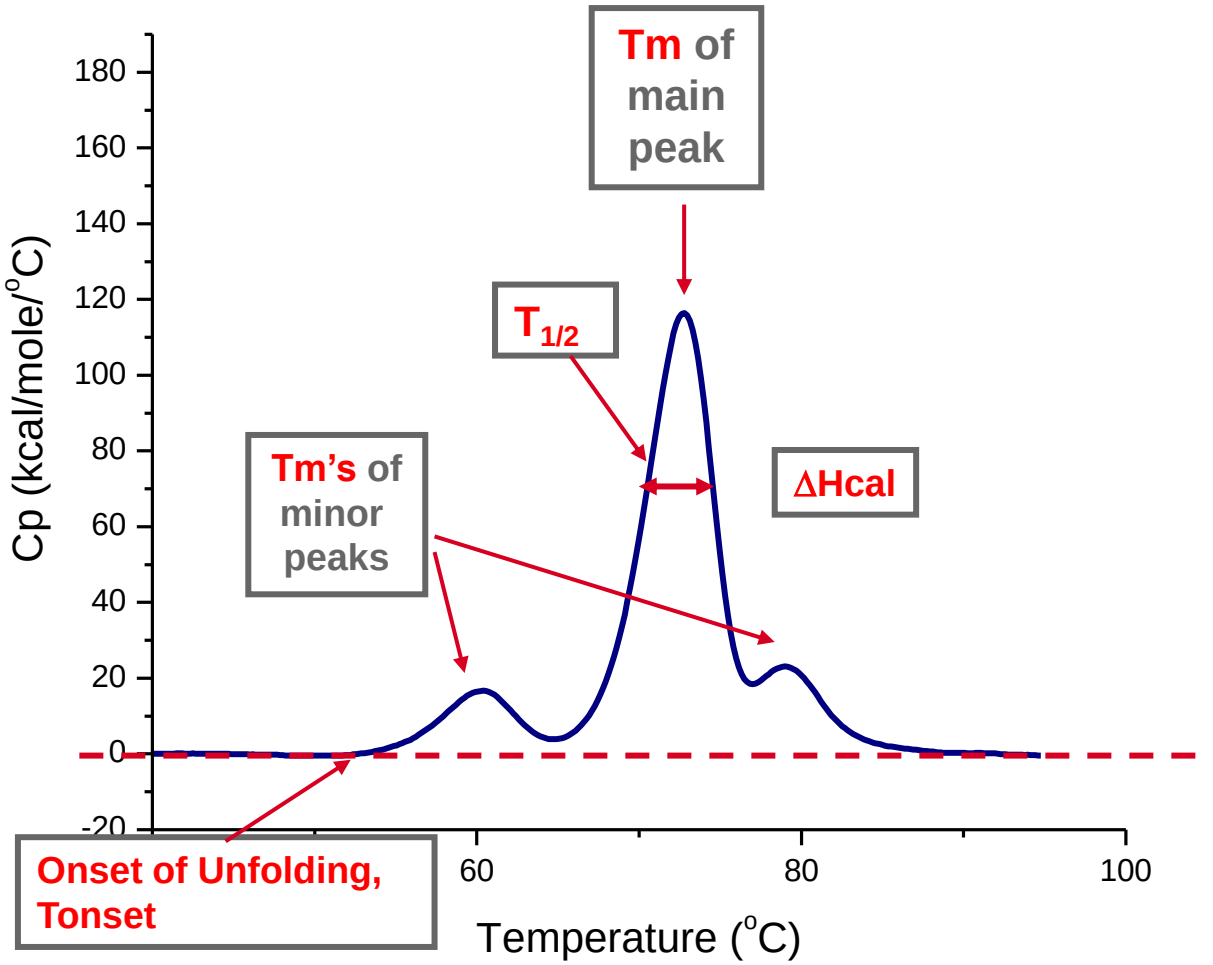
(I) Structural changes caused by conformational instability of protein native state.

(II) Propensity of a protein to form intermolecular bonds, assembly.

Adopted from Uchiyama, S. (2014) ***Biochim Biophys Acta.*** 1844, 2041

DSC basics. Analysis of *irreversible* thermal protein unfolding

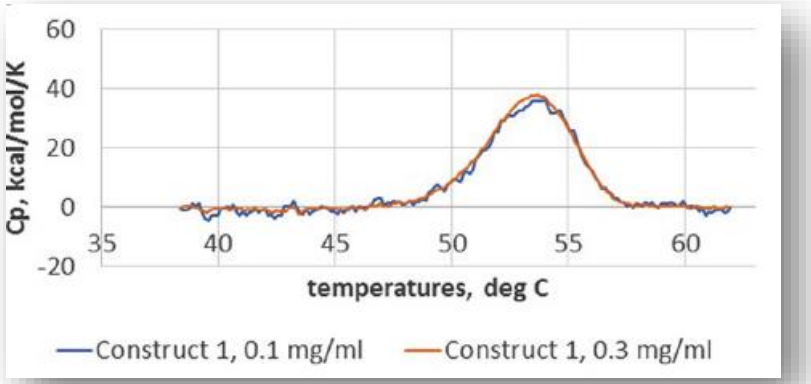
Protein stability on domain level (monoclonal antibody)

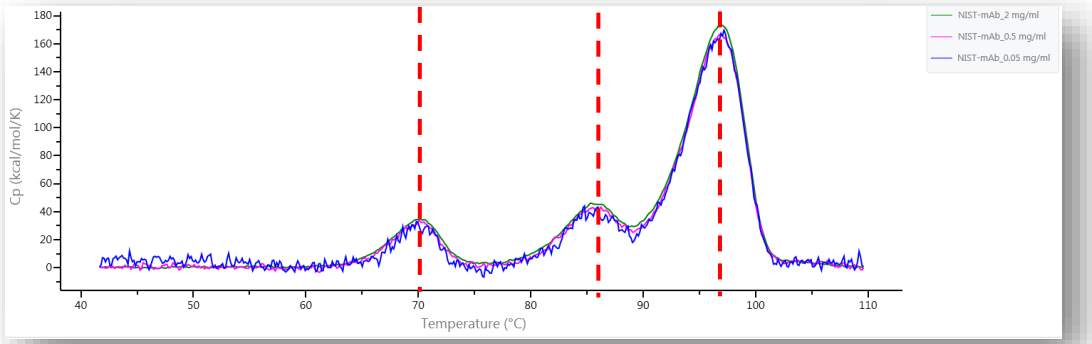


- Shifts in Tm values (°C):
  - Higher temperature = more stable
  - Lower temperature = less stable
- Shifts in Tonset values (°C):
  - Higher temperature = more stable
  - Lower temperature = less stable
- Lowering of "ΔH", area under the curve:
  - decrease in the content of folded protein
- Changes in $T_{1/2}$ values (transition width,°C):
  - Smaller width = more cooperative unfolding usually associated with a compact structure
  - Larger width – less cooperative usually associated with a relaxed, partially unfolded structure
- Scan rate dependence of unfolding transitions
  - DSC can be used to study kinetic aspect of protein stability, E_{act}

Different T_m at different protein concentrations indicative of protein oligomerization/ aggregation propensity

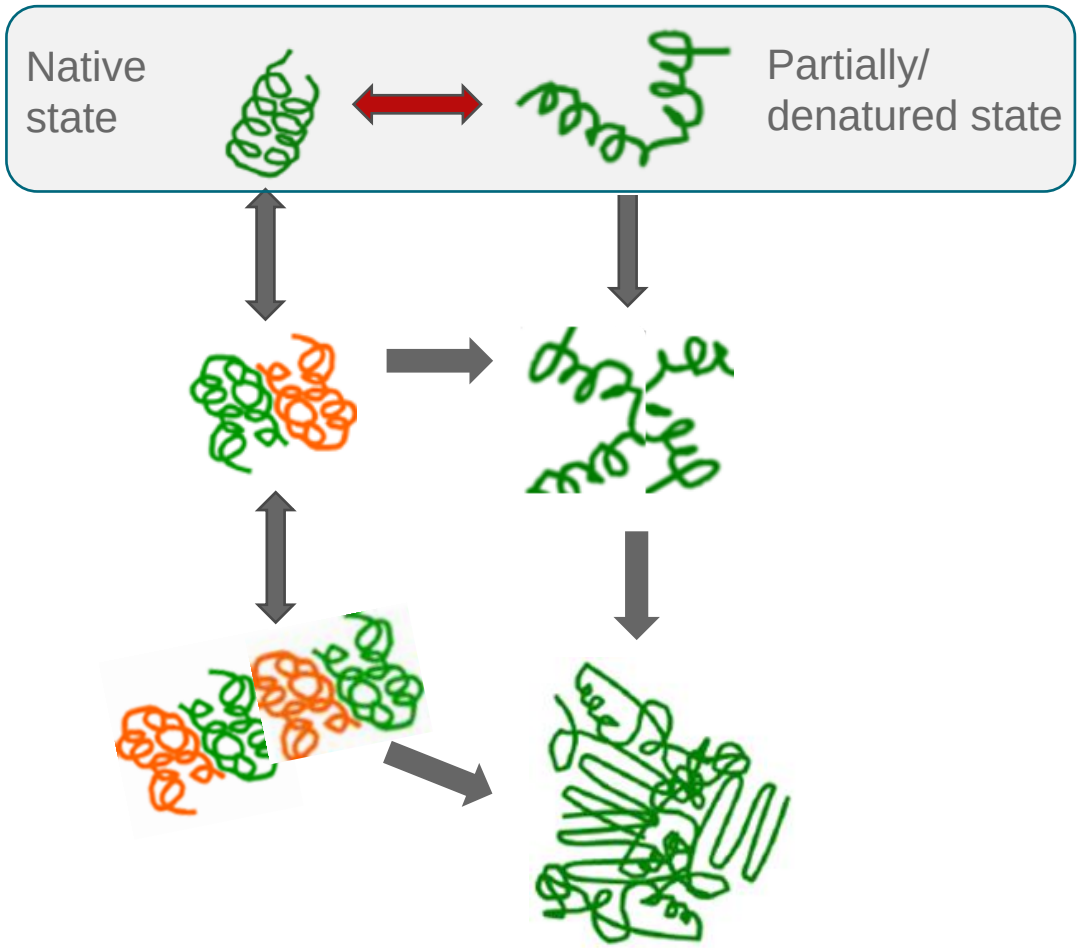
Self-interaction propensity probed at a series of proteins concentrations





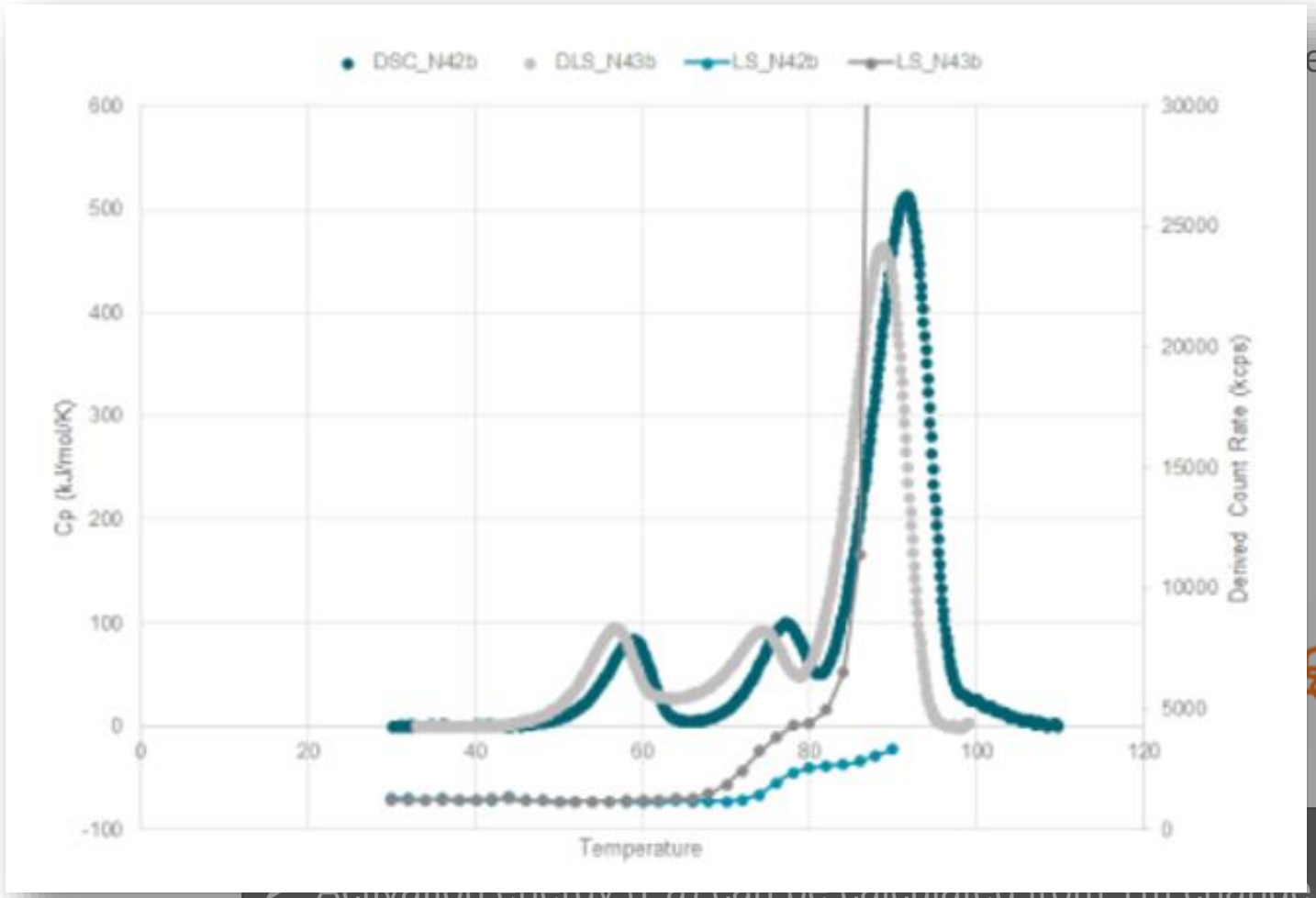
0.05; 0.5 and 2 mg/ml NISTmAb

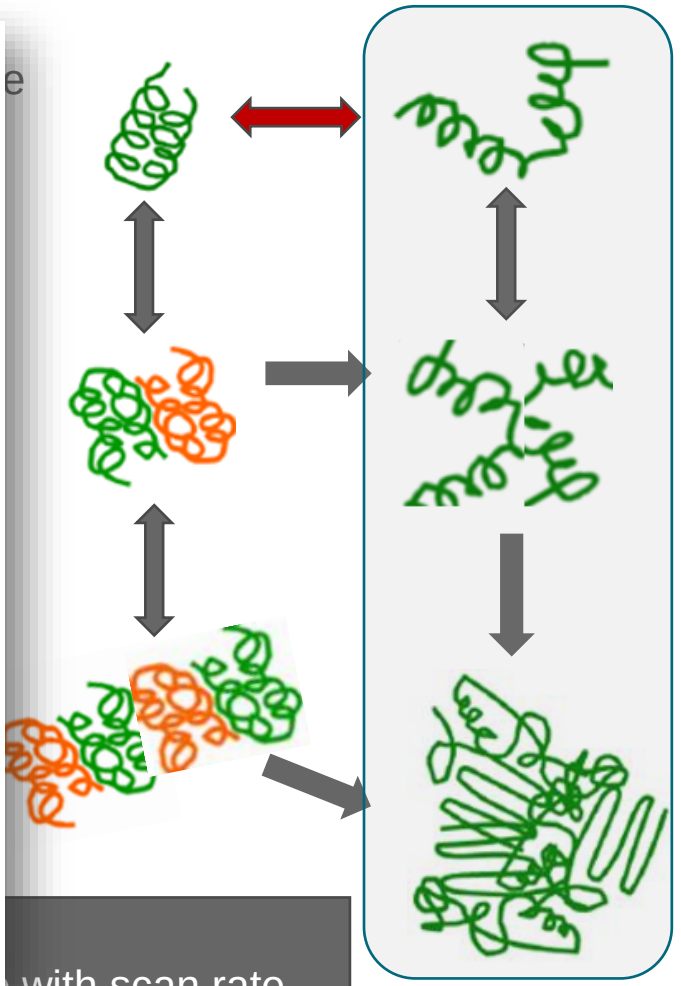
Tm may shift with increase of sample concentration
✓ Monomer Tm is concentration independent



Different T_m at different protein concentrations indicative of protein oligomerization/ aggregation propensity

Self-interaction propensity probed at a series of proteins concentrations





Partially/ denatured state

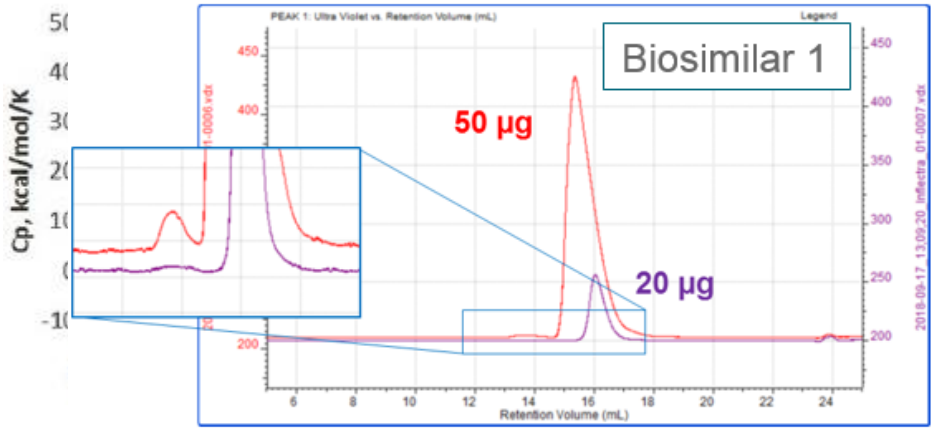
$N \rightleftarrows U \xrightarrow{k} D$

$N \xrightarrow{k} D$

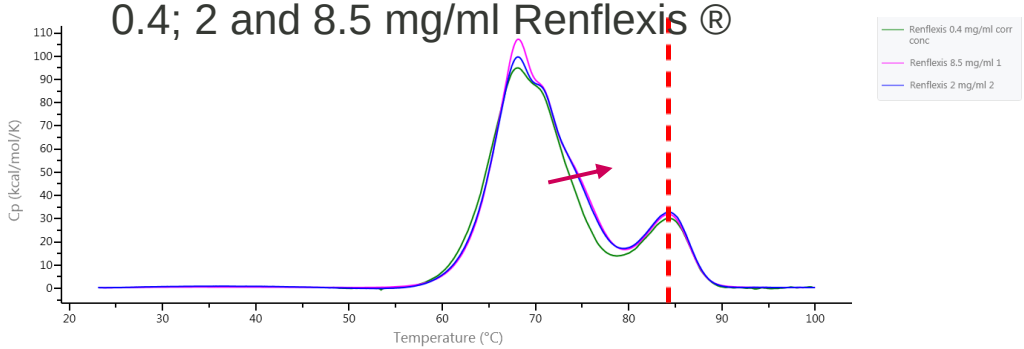
- Activation energy (Ea) can be calculated from Tm change with scan rate

Different T_m at different protein concentrations indicative of protein oligomerization/ aggregation propensity

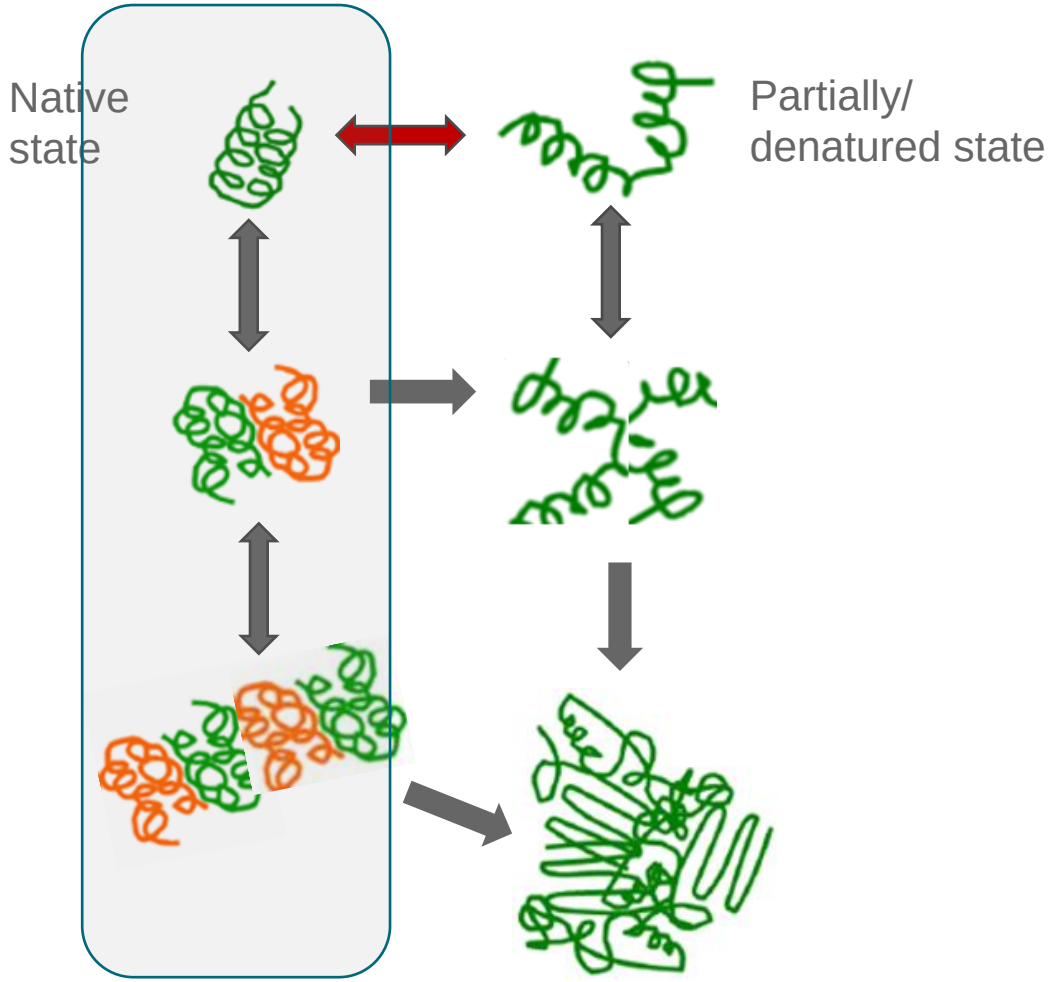
Self-interaction propensity probed at a series of proteins concentrations



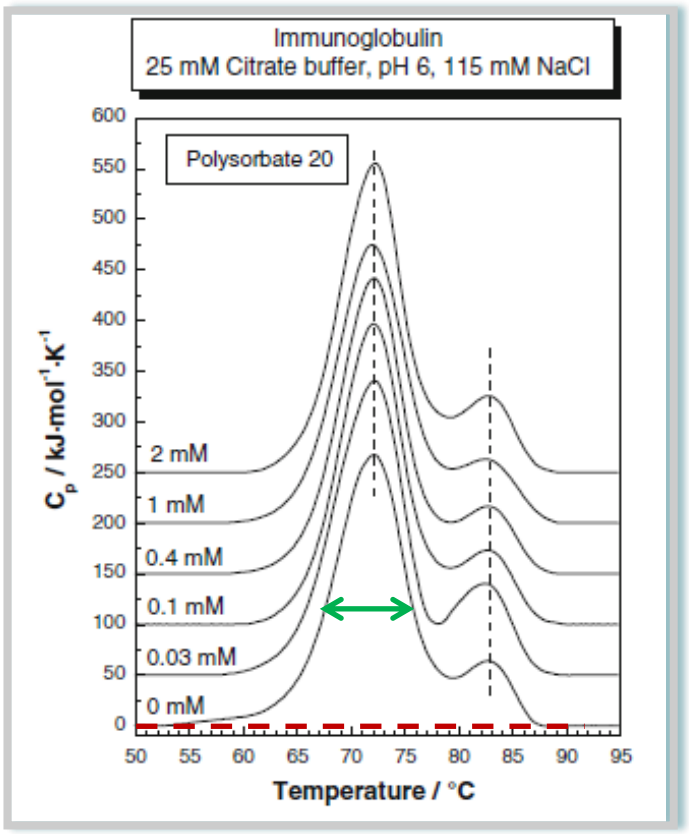
0.4; 2 and 8.5 mg/ml Renflexis ®



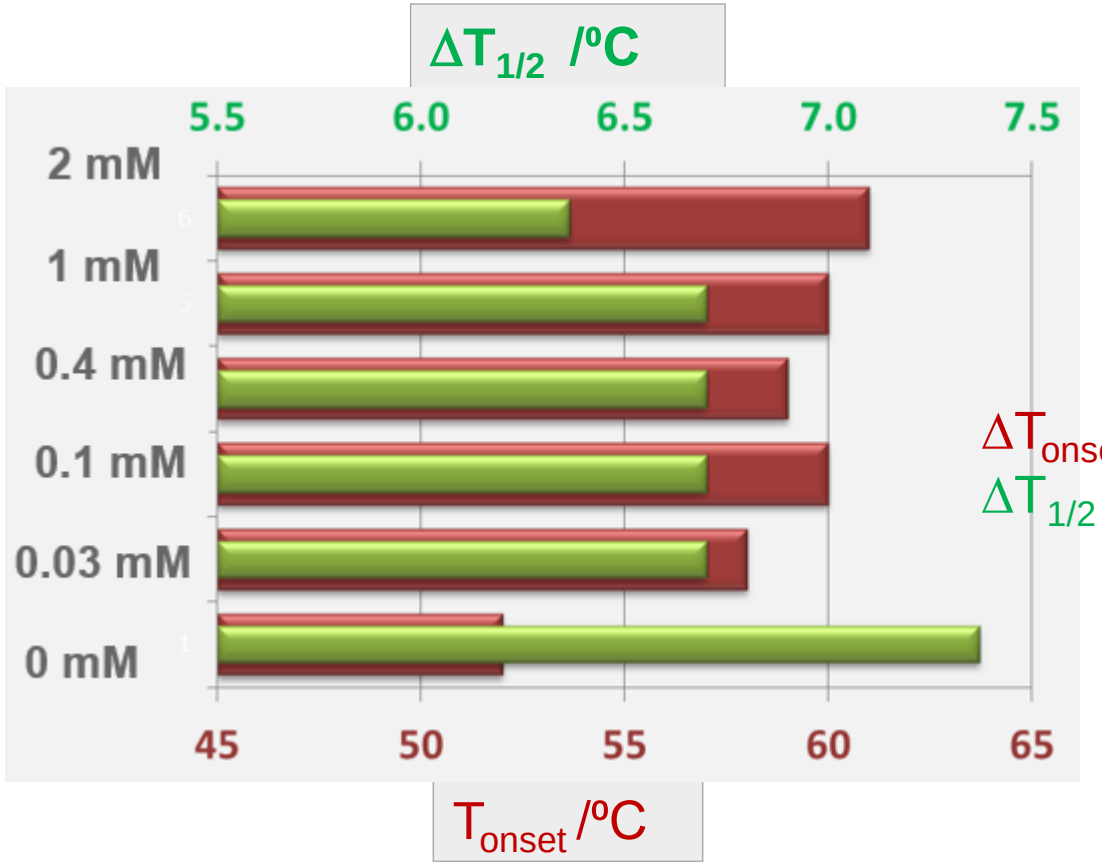
Tm may shift with sample concentration.
✓ Reversible oligomers give positive Tm shifts



Rationalizing effect of excipients. T_ms do not tell the whole story. Do look at multiple stability metrics



"No effect of Tween 20 on IgG detected by DSC"



ΔT_{onset} ~ 8°C
$\Delta T_{1/2}$ ~ 1.2°C

Tween 20 affects cooperativity of mAb unfolding based on multiple stability metrics obtained with DSC

Adopted from Hoffman et al. Eur. Biophys. J. (2009) 38: 557-568

Tm, $T_{1/2}$ and T_{onset} . Multiple metrics of protein thermal stability make pre/formulation funnel more efficient.

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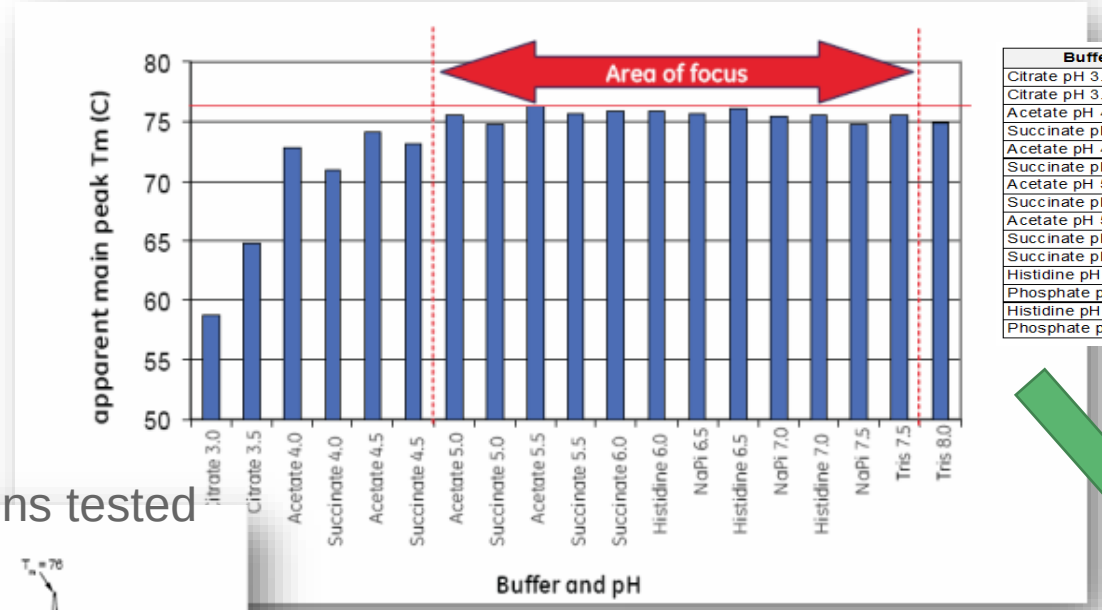
12 selected

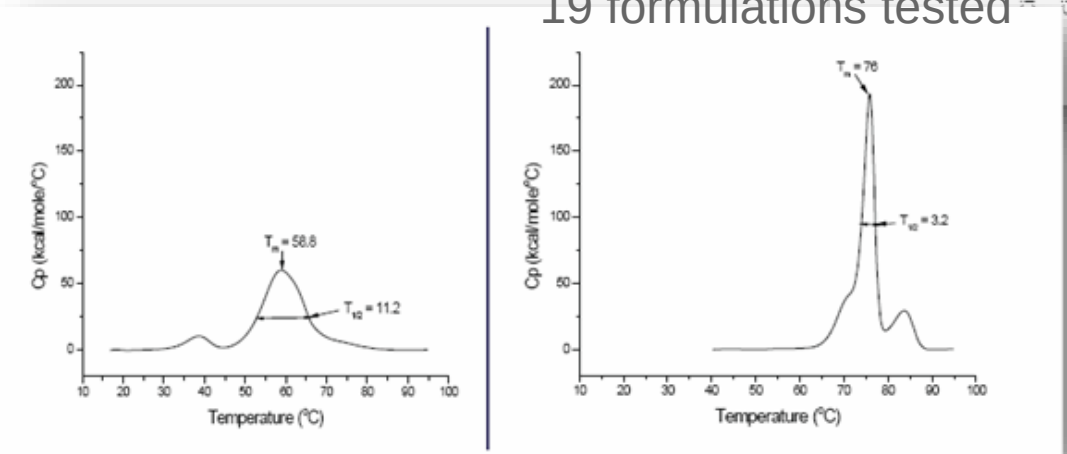


Buffer	Tm 1 (C_H2)	Tm 2 (main)	Tm 3 (C_H2)	Tonset	T1/2
Citrate pH 3.0	38.6	58.8	ND	29	11.2
Citrate pH 3.5	46.5	64.8	ND	41	8.0
Acetate pH 4.0	60.4	72.8	79.1	53	4.9
Succinate pH 4.0	56.2	71.0	76.6	50	5.7
Acetate pH 4.5	64.1	74.1	81.9	56	4.7
Succinate pH 4.5	61.2	73.1	79.7	53	5.2
Acetate pH 5.0	ND	75.6	83.1	62	4.0
Succinate pH 5.0	65.6	74.8	82.2	57	3.8
Acetate pH 5.5	ND	76.3	83.7	64	3.5
Succinate pH 5.5	ND	75.7	83.2	62	3.5
Succinate pH 6.0	ND	76.0	83.7	65	3.2
Histidine pH 6.0	ND	75.9	83.6	63	3.5
Phosphate pH 6.5	ND	75.7	83.6	66	3.2
Histidine pH 6.5	ND	76.0	83.7	65	3.5
Phosphate pH 7.0	ND	75.4	83.5	66	4.0

Rank by T_m

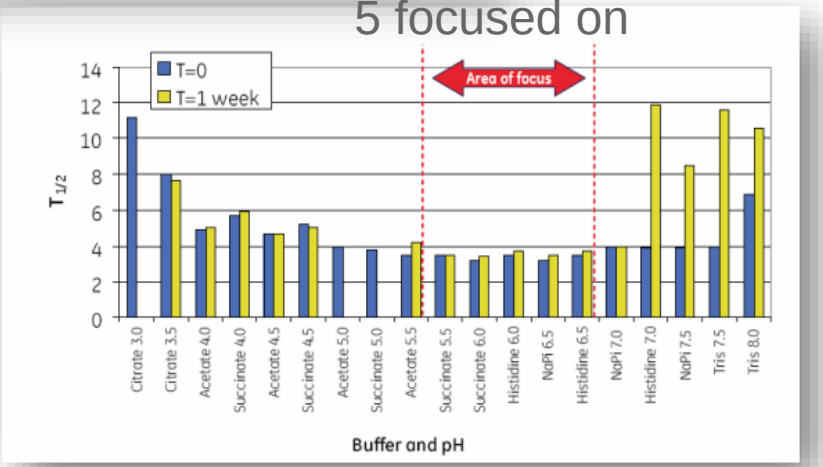
Focus with $T_{1/2}$

19 formulations tested



Katherine E. Bowers, Malvern Application Note

5 focused on



$\Delta H_{cal}/\Delta H_{vH}$ ratio as indicator of unfolding mechanism.

- $\Delta H_{vH} = \Delta H_{cal}$ – cooperative unit and molecular weight are the same: largely **reversible unfolding of one single domain**
- $\Delta H_{vH} < \Delta H_{cal}$ - cooperative unit is smaller than molecular weight: **intermediates**
- $\Delta H_{vH} > \Delta H_{cal}$ – cooperative unit is bigger than the molecular weight: **oligomers** or overestimated concentration of folded protein

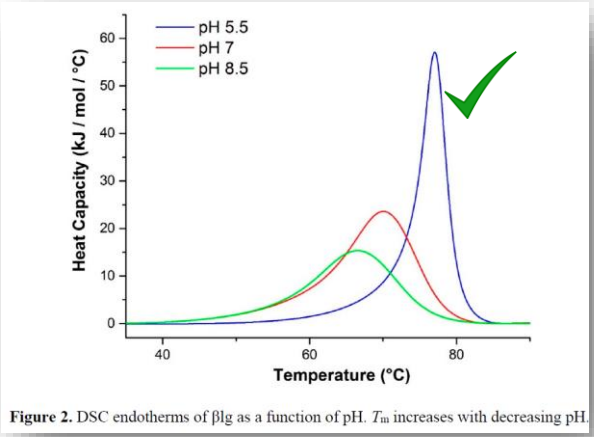


Figure 2. DSC endotherms of βlg as a function of pH. T_m increases with decreasing pH.

pH	T_m (°C) [a]	ΔH_v (kJ/mol) [a]	ΔH_{cal} (kJ/mol) [a]	ΔH_v [a]:ΔH_{cal} [a]
5.5	76.7	711.3	298.9	2.380
7.0	69.2	281.8	316.6	0.8901
8.5	66.0	250.1	232.0	1.078

Steven Blake et al. *Int. J. Mol. Sci.* **2015**, *16*

DSC extremely valuable and widely applicable biophysical method for therapeutic protein product development

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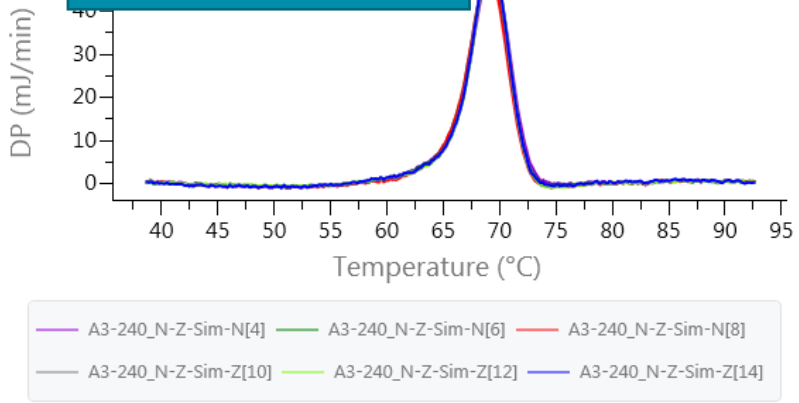
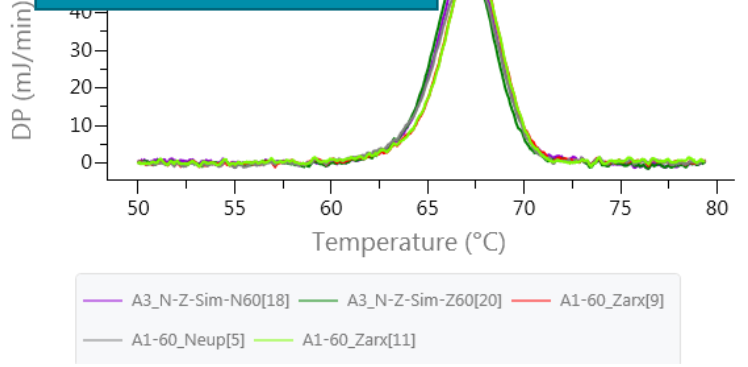
"Determining the thermal transition temperature of a protein has great utility in comparing the relative stabilities of candidate molecules for a given product, screening formulation conditions to optimize protein stability, comparing stability for a given product before and after a manufacturing change, and for comparing a biosimilar to an innovator product.
With MicroCal VP-Capillary DSC**, these types of studies can be performed with minimal hands-on work by the operator. **The results obtained are by far the most reproducible of any I have seen from any analytical instrument. Incredibly, thermograms from a half dozen analyses of a given sample can be overlaid and one cannot see anything but a single line. Amazing!"

Prof John Carpenter
Dept Pharmaceutical Sciences
UC Denver

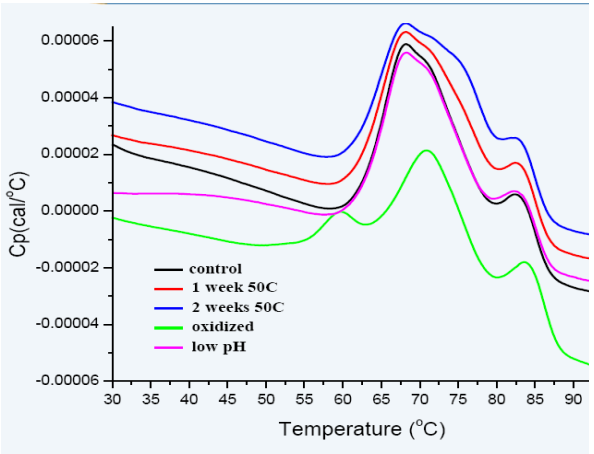
Intra-instrument biosimilarity test



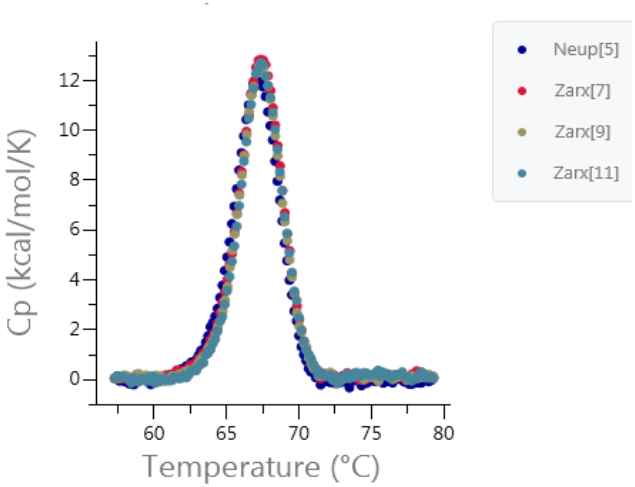
Inter-instrument biosimilarity test



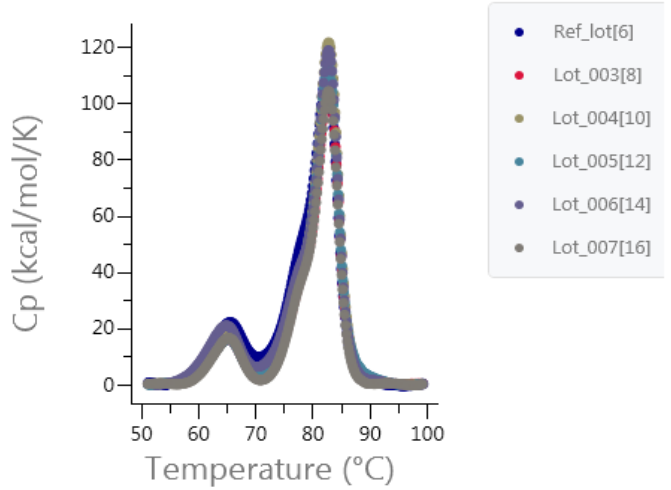
DSC thermogram as fingerprint of protein unfolding and stability.



HOS: DSC thermograms of a mAb before and after forced degradation. (From Malvern webinar W151022).



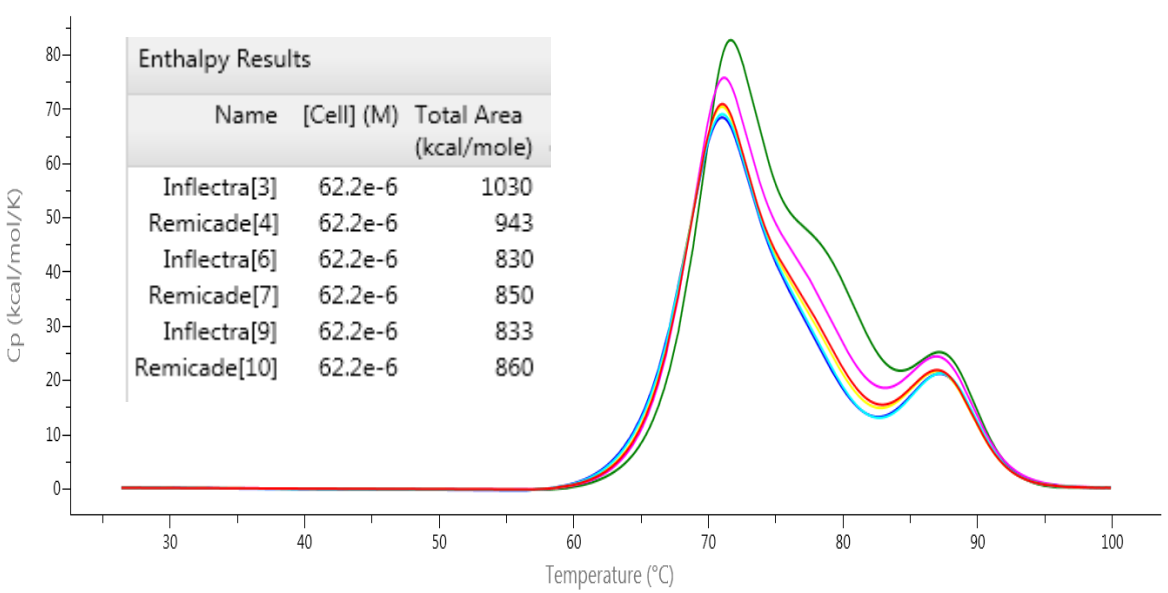
Biosimilarity: DSC thermograms of Neupogen (innovator) compared to Zarxio (biosimilar). (Samples from J. Carpenter, U. Colorado).



Comparability: DSC thermograms of mAb reference lot compared to five other lots of same mAb. (Data from Malvern Instruments).

Best practices

Need for extra rigorous cleaning



Record Name	Tm_1 (°C)	$TmHalf_1$ (°C)	ΔH_1 (kcal/mol)	ΔHVH_1 (kcal/mol)	TOnset
Inflectra[3]	71.59	10.62	0	0	62.03
Remicade[4]	71.11	9.70	0	0	61.35
Inflectra[6]	70.92	8.93	0	0	60.62
Remicade[7]	70.92	9.08	0	0	61.02
Inflectra[9]	70.93	8.97	0	0	60.76
Remicade[10]	70.98	9.21	0	0	61.08

- Compared to the 1st protein scan, scans 2-6 have a lower area and Tm1 is about 0.5 higher for scan 1.
- For these samples under these conditions the shorter detergent wash was not sufficient to completely clean the PEAQ-DSC cells.

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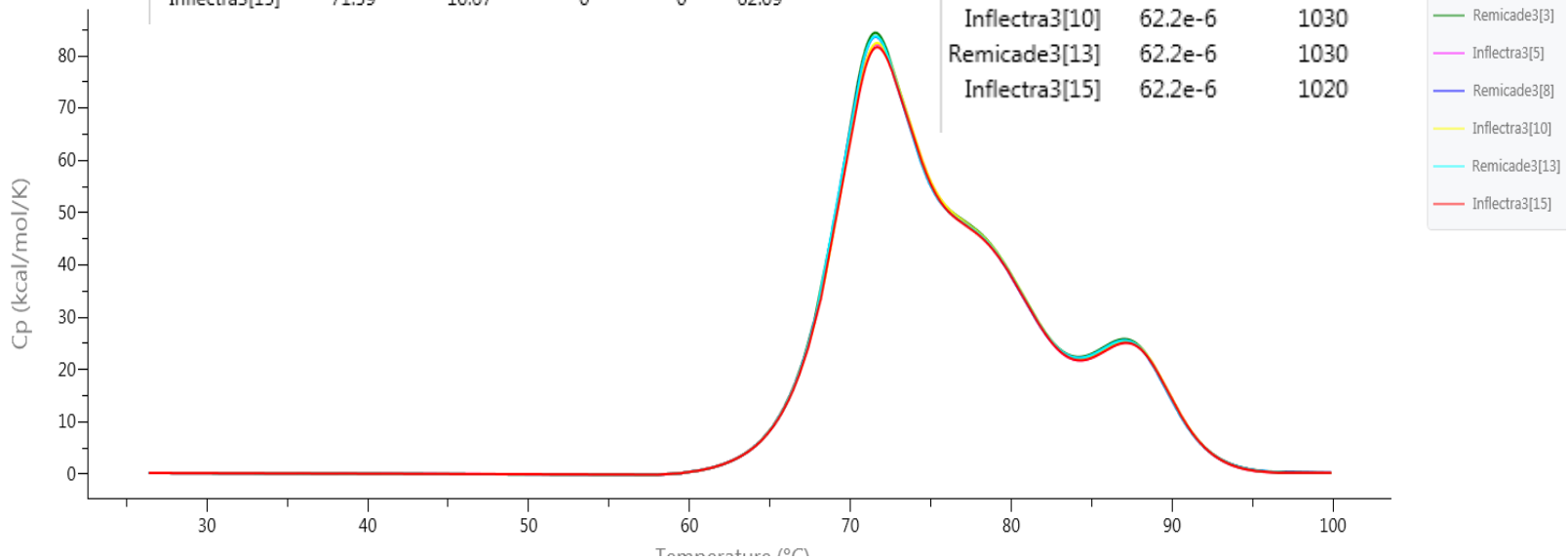
Best practices

Effect of adequate cleaning. Excellent repeatability.

Record Name	Tm_1 (°C)	$TmHalf_1$ (°C)	ΔH_1 (kcal/mol)	ΔHVH_1 (kcal/mol)	TOnset
Remicade3[3]	71.52	10.48	0	0	62.09
Inflectra3[5]	71.59	10.64	0	0	62.03
Remicade3[8]	71.53	10.47	0	0	62.10
Inflectra3[10]	71.60	10.67	0	0	62.03
Remicade3[13]	71.51	10.48	0	0	62.07
Inflectra3[15]	71.59	10.67	0	0	62.09

Enthalpy Results

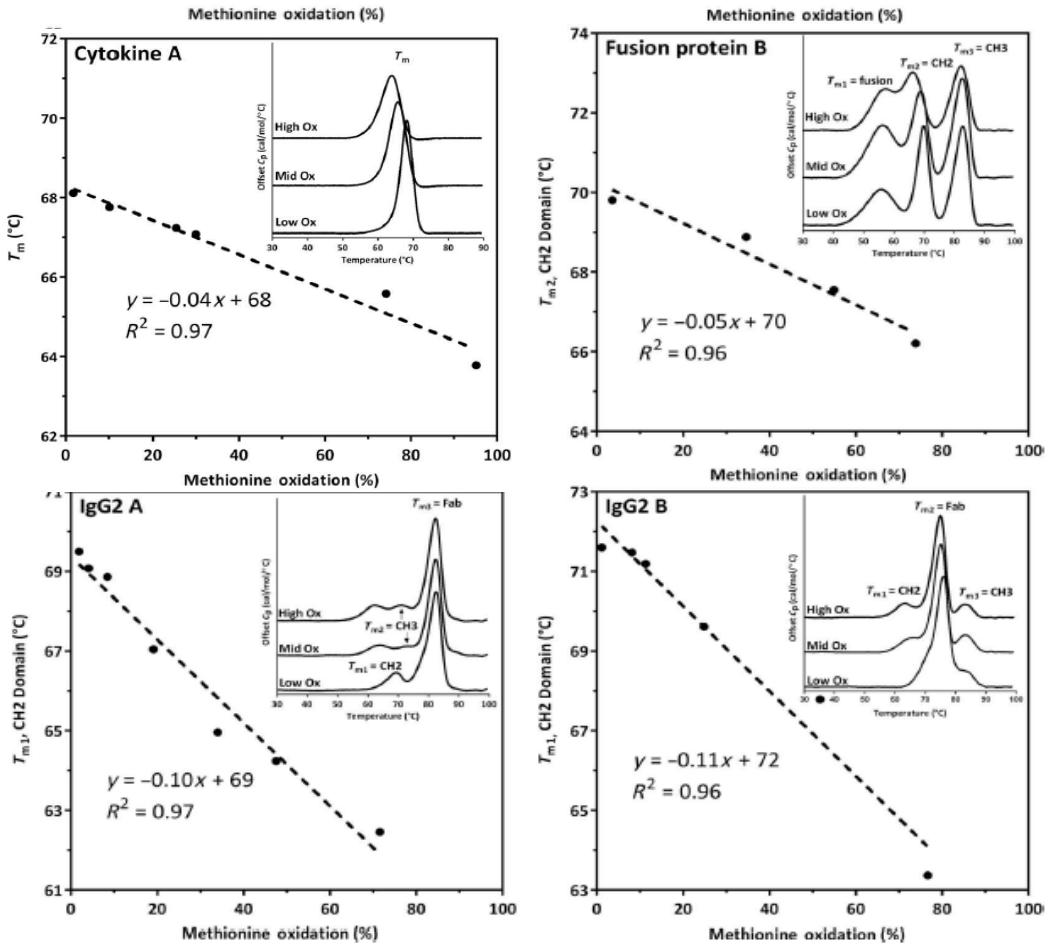
Name	[Cell] (M)	Total Area (kcal/mole)
Remicade3[3]	62.2e-6	1040
Inflectra3[5]	62.2e-6	1020
Remicade3[8]	62.2e-6	1030
Inflectra3[10]	62.2e-6	1030
Remicade3[13]	62.2e-6	1030
Inflectra3[15]	62.2e-6	1020

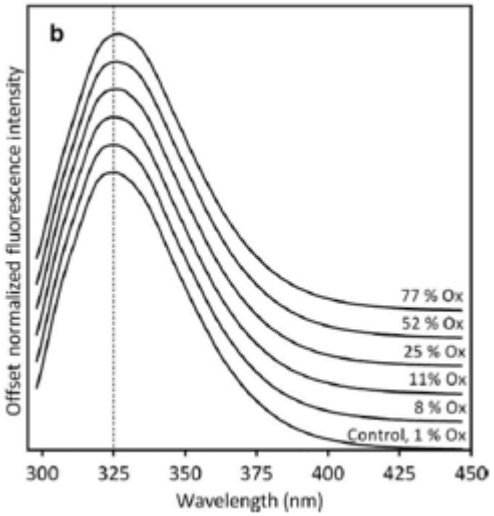


- Detergent scan after every sample scan ensures adequate cleaning and results in identical sample scans

DSC for monitoring HOS changes or deterioration in a biotherapeutic product

Chemical instability



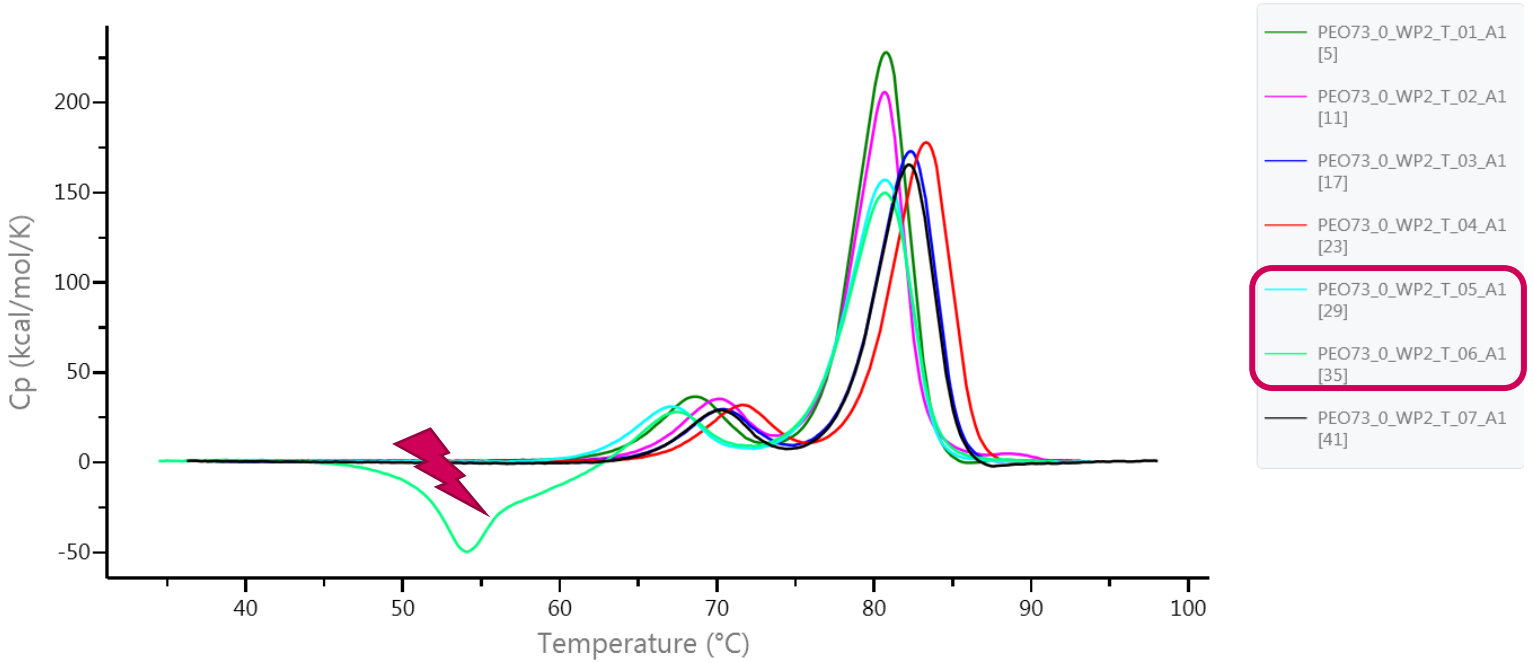


Arthur et al, J Pharm Sci, 104,1548–1554 (2015)

- ✓ **DSC consistently showed enhanced sensitivity to structural changes compared to spectroscopic based methods and can be a leading indicator of biological activity changes prior to detection by potency assays.**
- ✓ **DSC is sensitive and reproducible to assess product quality and investigate OOS and OOT.**

And more!

DSC can spot chemical reactivity, e.g. reactivity of excipients used in formulation development



- Oxidative species may be implied in the long-term oxidative degradation
- Antioxidants might act as pro-oxidants in metal-catalyzed oxidation
- Controlling metal ion contamination level in the excipients and limiting available molecular oxygen are recommended for formulation development

Case study.
Stability profiling and formulation development for a fusion protein X.
In collaboration with Cobra Biologics

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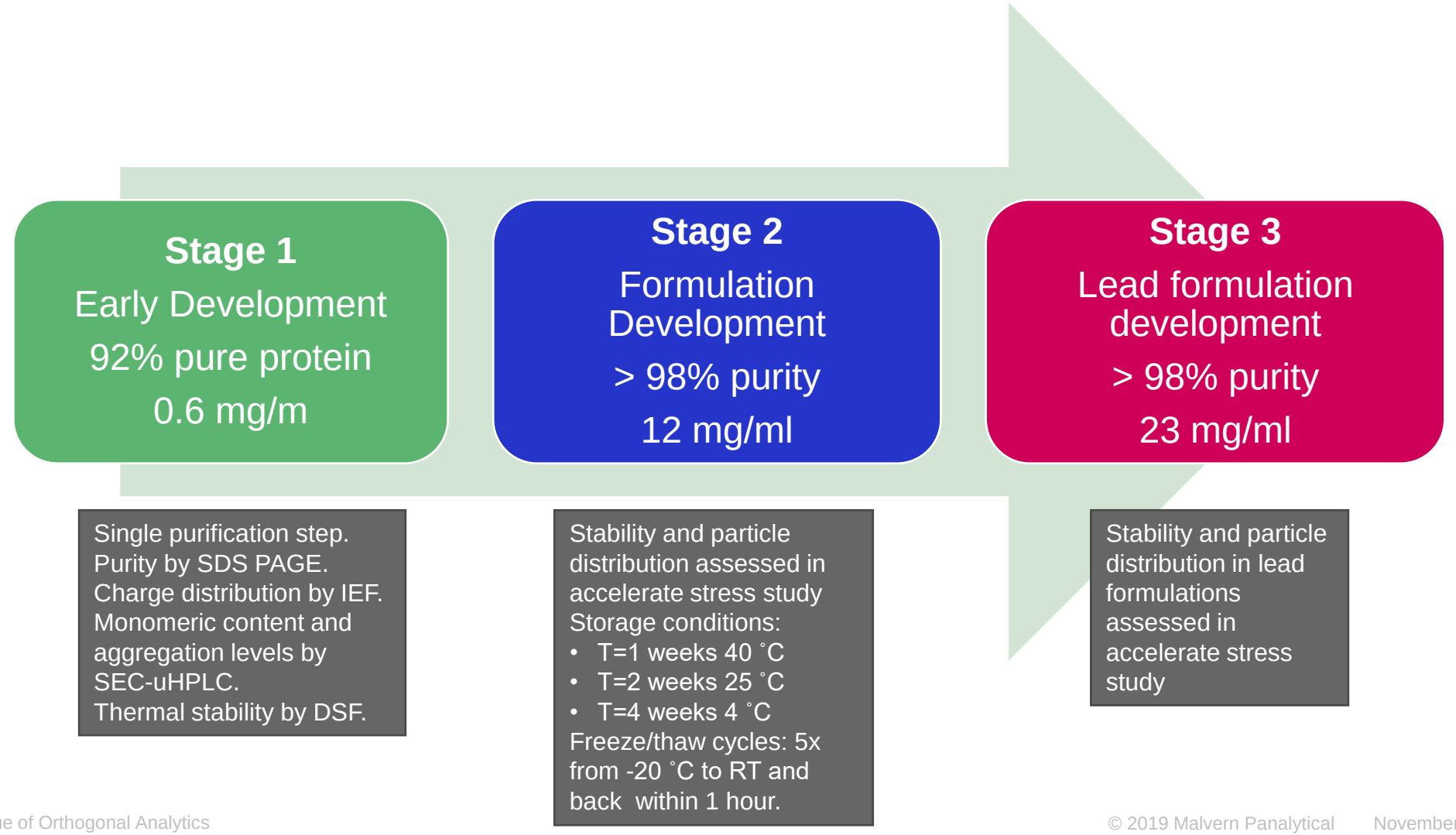
Project Analytics and Development Workflow at customer site



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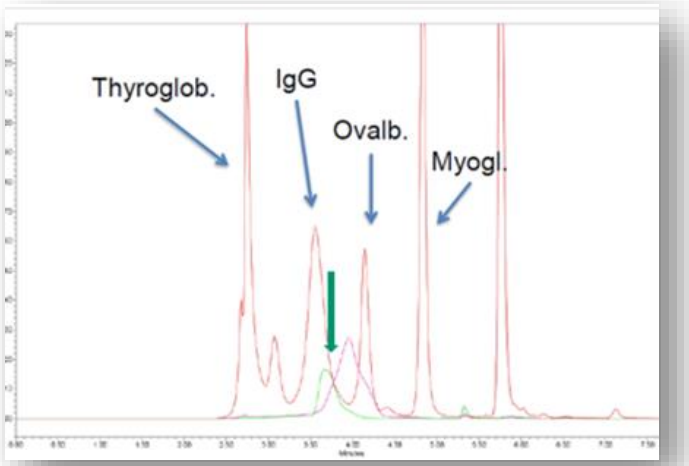
Project results delivered by the platform technologies

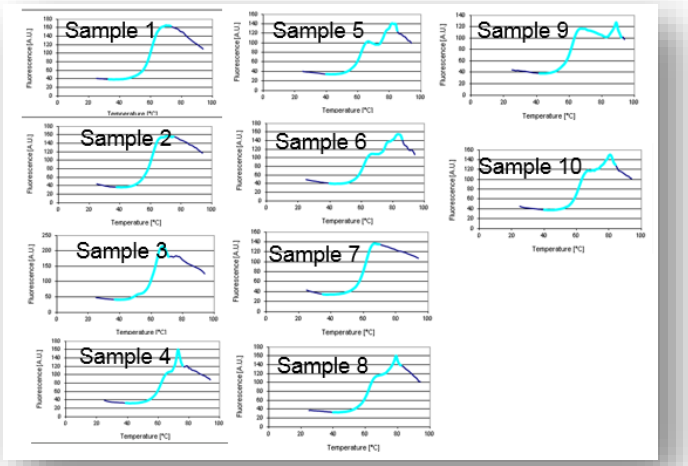
Findings and open questions



STATE IN SOLUTION

SEC-uHPLC UV

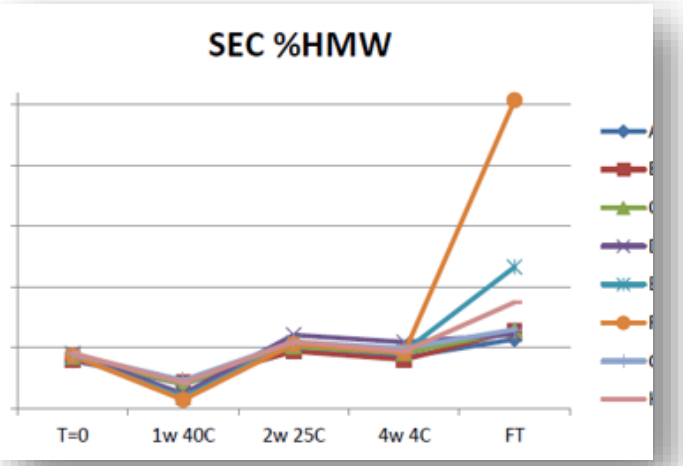
- What are the species eluting in the main peak?



THERMAL STABILITY

DSF

- How to rank stability by complex unfolding profile when 1st transition show minimal change?
- What is the significance of the second transition observed in 7 out of 10 buffers?



ACCELERATED STRESS STABILITY

SEC-uHPLC UV

- Can the accelerated stress stability data be rationalized in terms of intrinsic properties of Protein X?
- What are the species and mechanisms involved in degradation pathways of Protein X and how to affect them?

Bridging the gaps of the platform technologies

1st principle orthogonal biophysical techniques

MicroCal PEAQ-DSC
325 µl/sample at 0.5 and 5 mg/ml

- Protein X (M_W 62 kDa)
- All samples buffer exchanged and supplied with matching buffers

Zetasizer DLS
20 µl 0.6-23 mg/ml

MicroCal PEAQ-ITC
65 µl at 23 mg/ml

OMNISEC
20 µl at 0.6, 12 and 23 mg/ml

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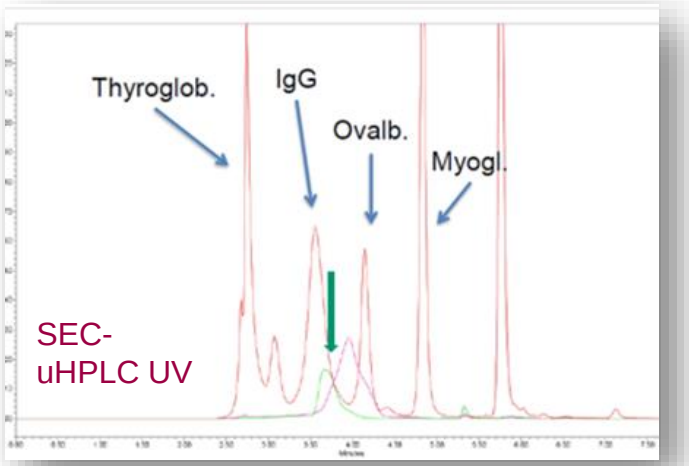
Stability Profiling of Protein X

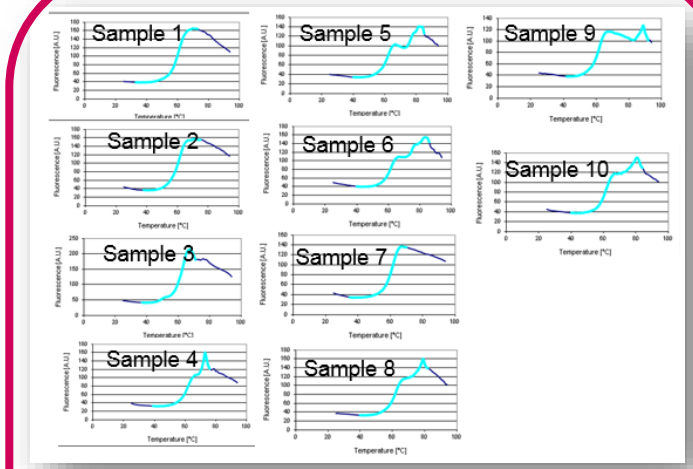
Findings and open questions



STATE IN SOLUTION

Multiple-detection SEC

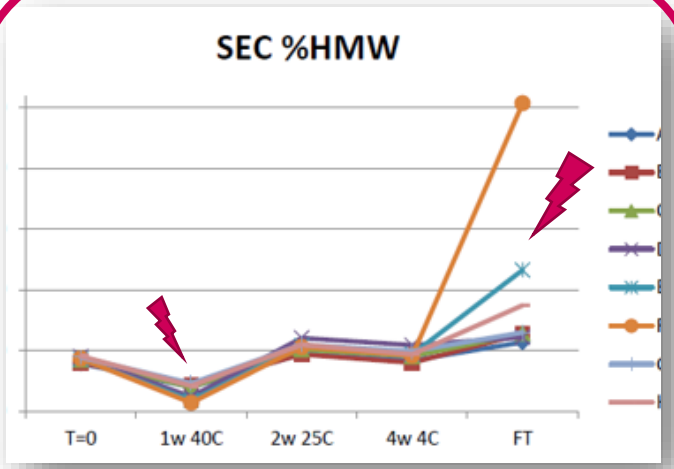
- ✓ Monomer species are eluting as main peak.
- ✓ Dimers represent predominant oligomer fraction followed by trimers and HMWA.
- ✓ Fraction of dimers increases with Protein X concentration



THERMAL STABILITY

DSF

- How to rank stability by complex unfolding profile when 1st transition show **minimal change**?
- What is the **significance of the second transition** observed in 7 out of 10 buffers?



ACCELERATED STRESS STABILITY

SEC-UHPLC UV

- Can the accelerated stress stability data be rationalized in terms of **intrinsic properties of Protein X**?
- What are the species and mechanisms involved in **degradation pathways** and how to affect them?

Project Gain with DSC

Reliable artifact-free information on thermal stability

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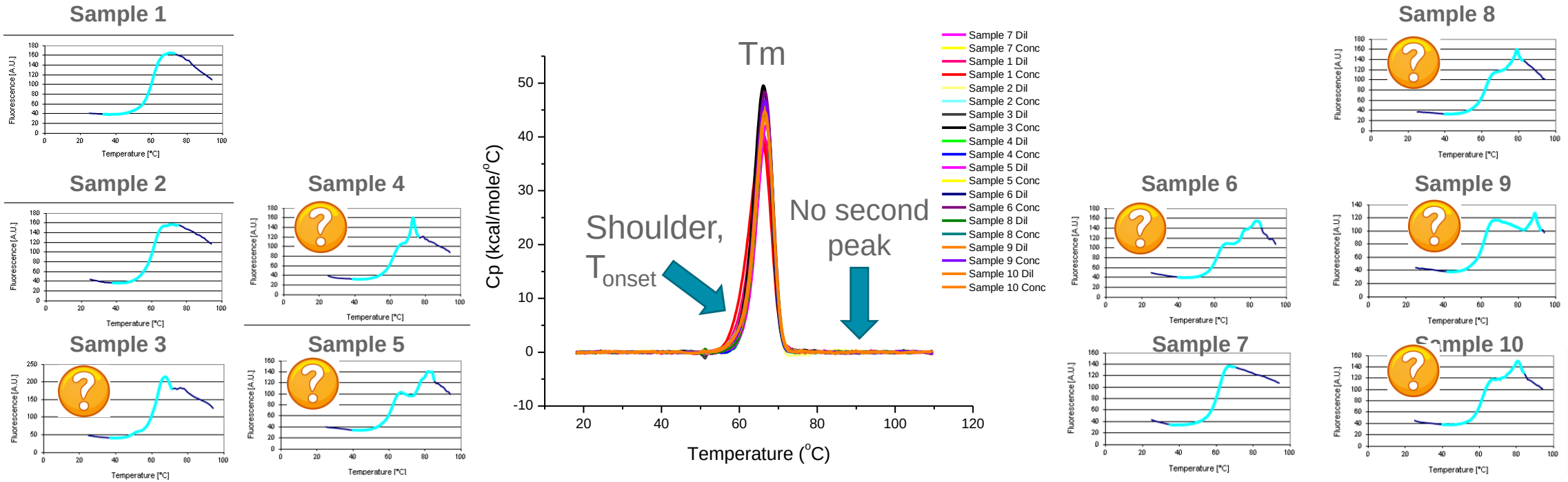
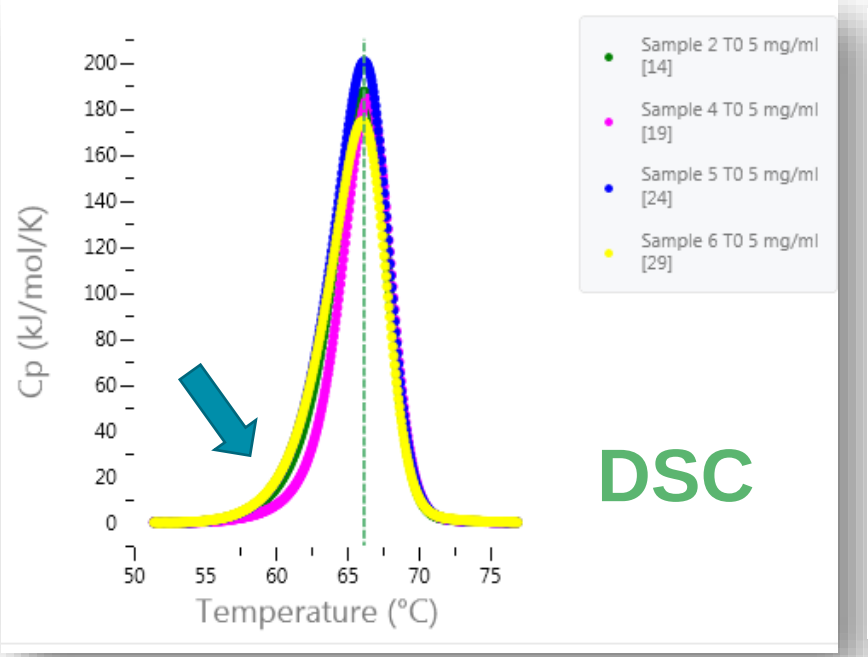


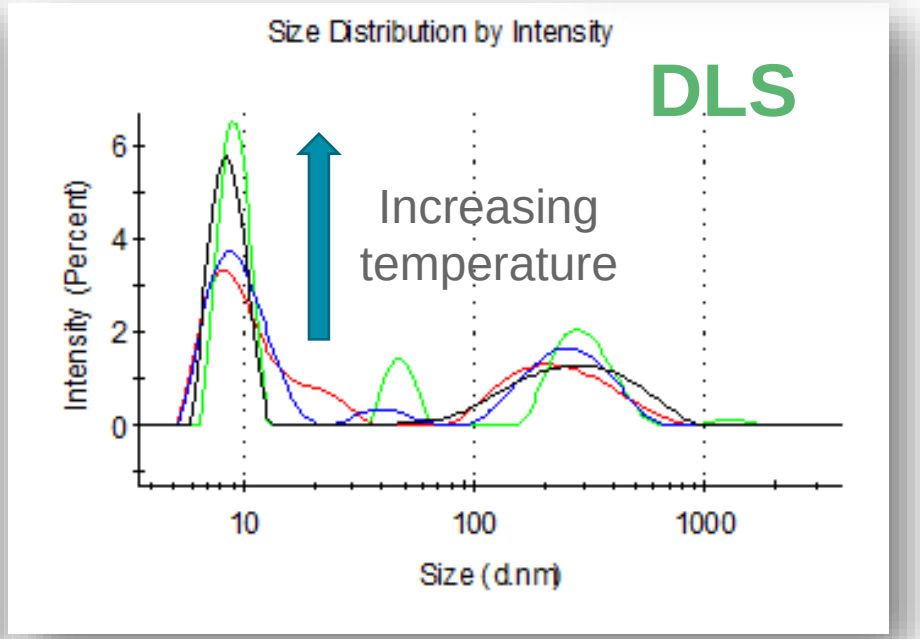
- Thermal unfolding of Protein X appears as one peak
- T_m and $T_{1/2}$ show minimal variability with buffer conditions and Protein X concentration
- Low temperature shoulder gets pronounced with increase of Protein X concentration and T_{onset} varies with buffer conditions.

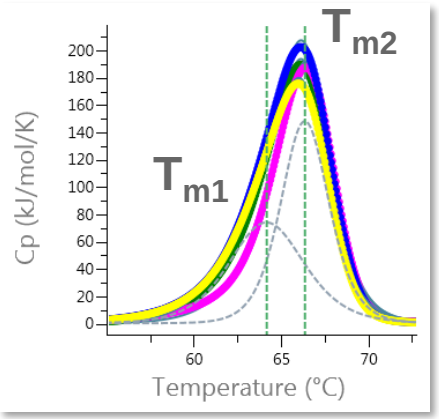
Could the shoulder reflect presence of several domains
and/or an additional process preceding and partly overlapping with protein unfolding?

Project Gain with DSC and DLS

What is causing the shoulder on DSC thermogram of Protein X?



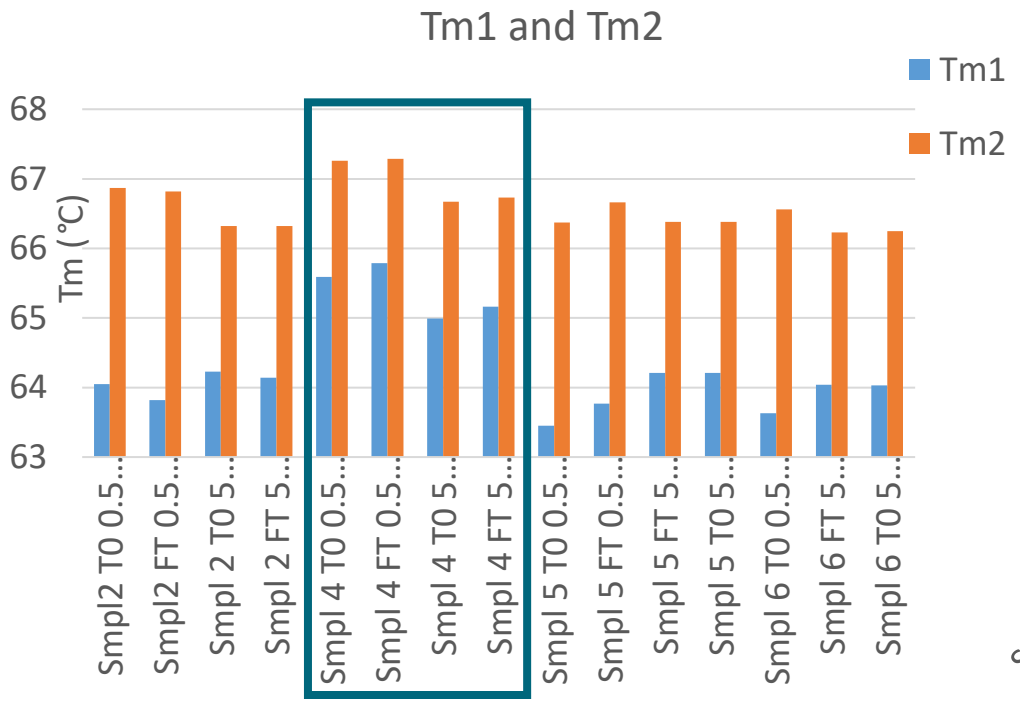


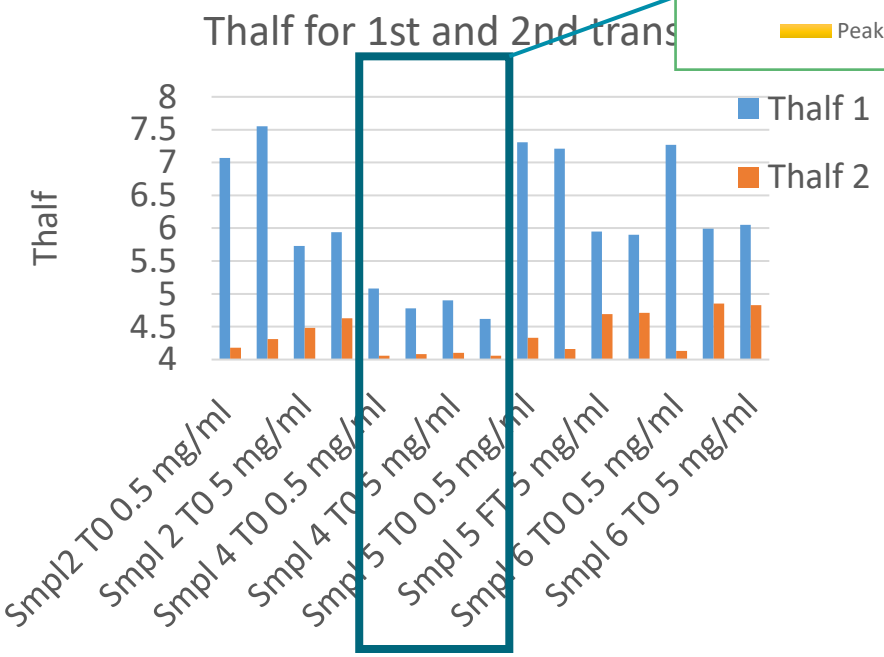


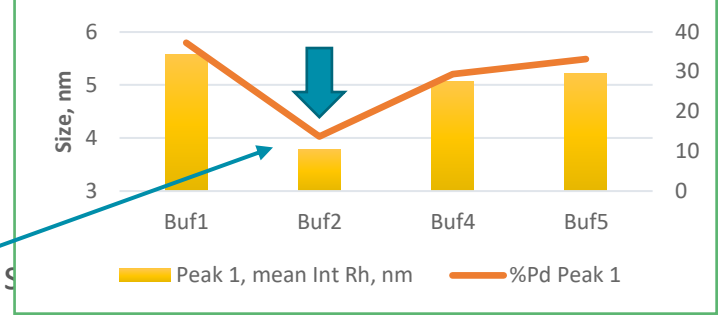
- Temperature-induced dissociation of oligomers confirmed by DLS data
- Shoulder on DSC thermogram of Protein X may reflect oligomer dissociation preceding and overlapping with protein unfolding

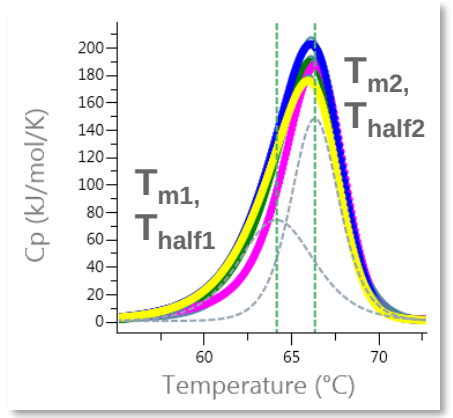
Project Gain. Complementarity of DSC and DLS

Thermal stability beyond T_m







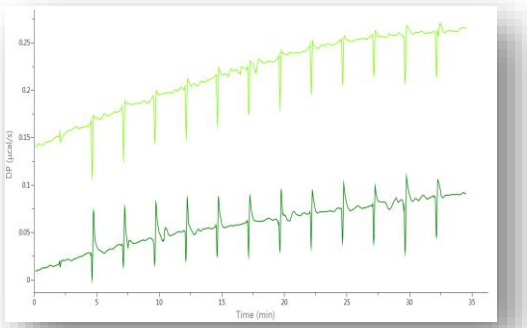


- T_{m1} and $T_{1/2}{}^1$ show highest sensitivity to sample conditions with variability of ≈3ºC as compared to ≈1ºC for variability in T_{m2} and $T_{1/2}{}^2$
- DSC points to conditions with highest thermal stability at Time = 0 and after 5 freeze-thaw cycles
- Finding confirmed by DLS

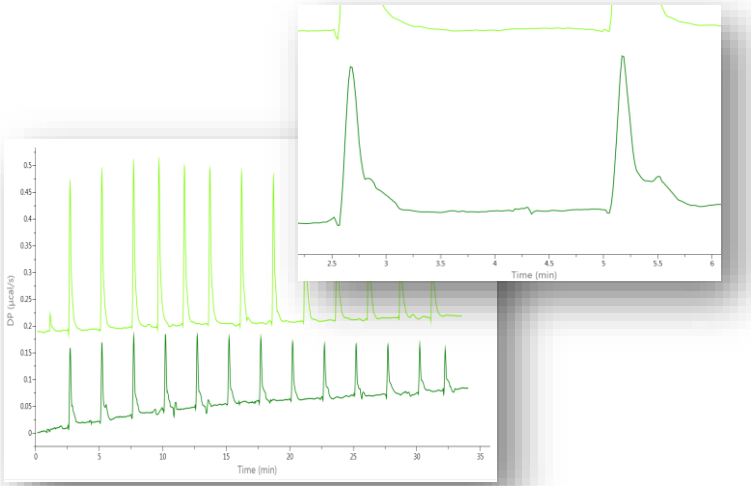
Project Gain with ITC

Self-association dynamics and kinetics of Protein X

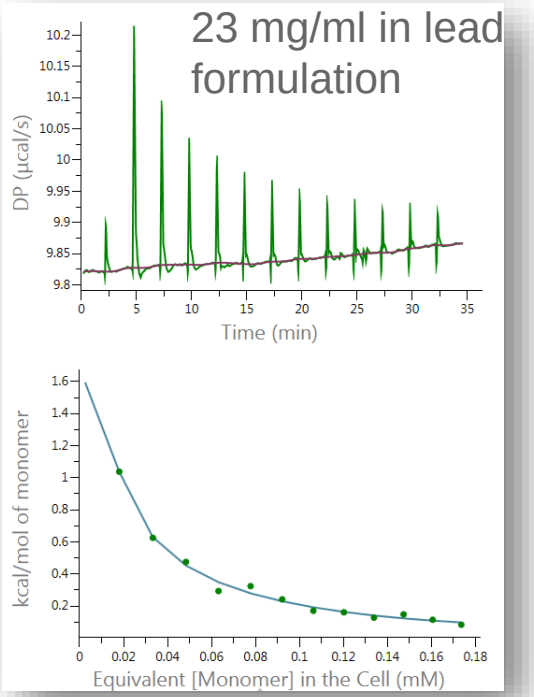
2 mg/ml and buffer control



21 mg/ml in formulation A and B



23 mg/ml in lead formulation

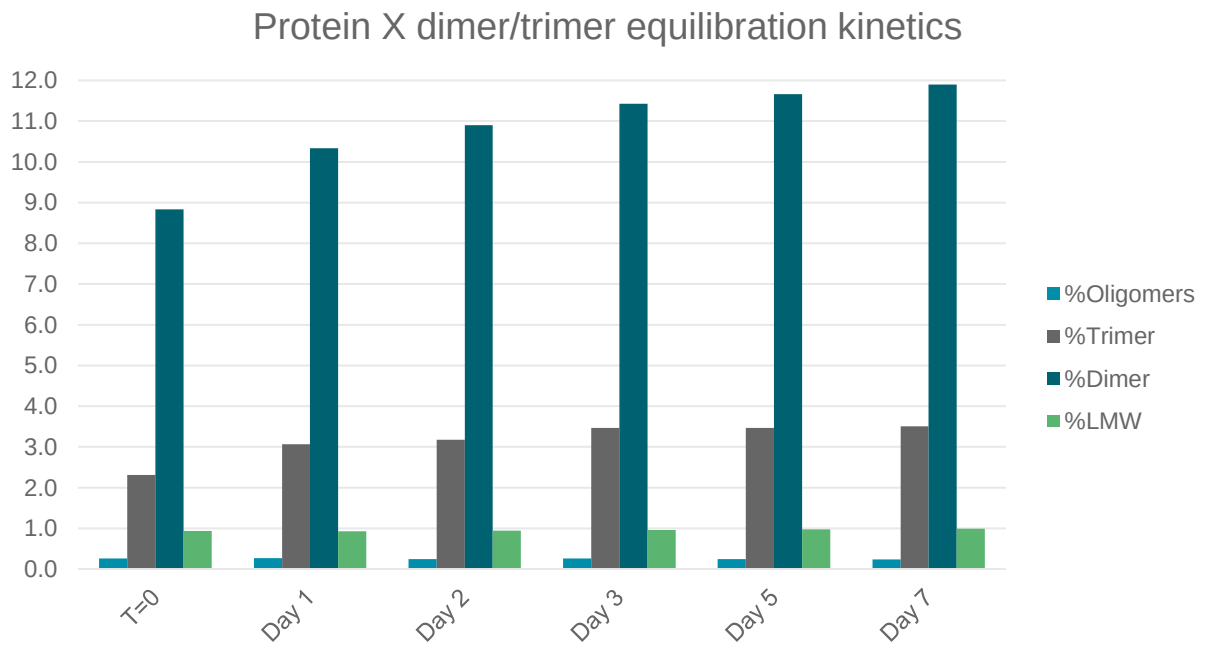


- Oligomer dissociation is kinetically controlled and proceeds at different rates dependent on buffer and storage conditions.
- Dilution factors, incubation time and temperature are critical to the state and stability of Protein X in solution

Kinetically controlled oligomerization of Protein X confirmed

Implications for analytical method development

- Protein X reference material was transferred from -70 °C to 4 °C on different days prior to analysis.
- The equilibrium was reached after 3 to 5 days at 4 °C.



Stability Profiling and Formulation for Protein X

DSC, OMNISEC, DLS and ITC. Sum is greater than its parts!

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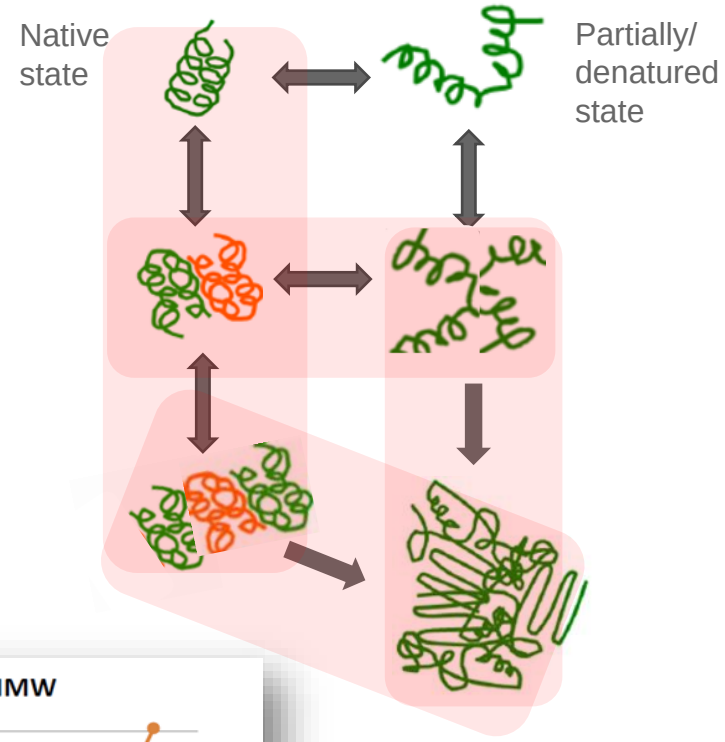
Multiple stability metrics by DSC

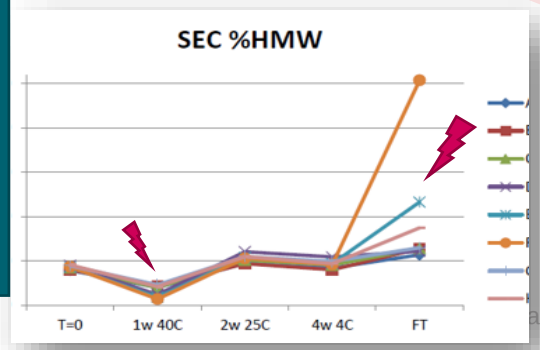
- informed selection of optimal formulations
- hinted on the complexity of the thermally induced transitions

Orthogonal use of DSC, DLS, multiple-detection SEC and ITC

- gave insights into intrinsic properties and behavior of Protein X in solution
- helped to rationalize degradation upon F/T cycles
- flagged for implications to analytical method development

- ➢ Increase of temperature and dilution triggers dissociation of oligomers of Protein X.
- ➢ Dimers/trimers of Protein X are favored by low temperature and are kinetically controlled.
- ➢ Oligomerization during freeze-thaw cycle conditions leads to formation of HMW aggregates



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