

# Protein stability issues in drug discovery

*Can biophysical techniques make a difference?*

SwedenBio Drug Discovery Workshop  
September 22, 2008

# Biophysical techniques



CD spectropolarimeter



DLS plate reader

DLS batch



Microcal ITC



Microcal DSC



600 MHz Varian Unity NMR Spectrometer  
equipped with cold probe, Gilson robot with Peltier cooling element, flow cell

Mass spectrometry  
MALDI-TOF MS,  
ESI-MS

Microcalorimetry  
ITC, DSC

Light Scattering

Analytical  
Ultracentrifugation

NMR

CD  
spectropolarimetry

SEC

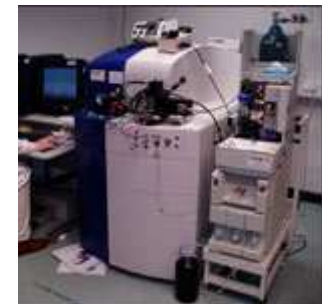
SPR



Thermal Activity Monitor



Biacore S51



Q-ToF Ultima API  
mass spectrometer



Analytical ultracentrifugation



MALDI-TOF mass spectrometer

# Biophysical techniques in drug discovery

- ✓ Medium to low throughput
- ✓ Generally larger amounts of protein needed as compared to biochemical assays

**So... why  
to bother?**



# Biophysical techniques in drug discovery



**I know the way through the  
mindfield, step exactly where I do.**

**Data on biochemical activity alone might be  
insufficient to guide protein purification  
process and to address the issues of  
irreproducible assay results or low protein  
activity**

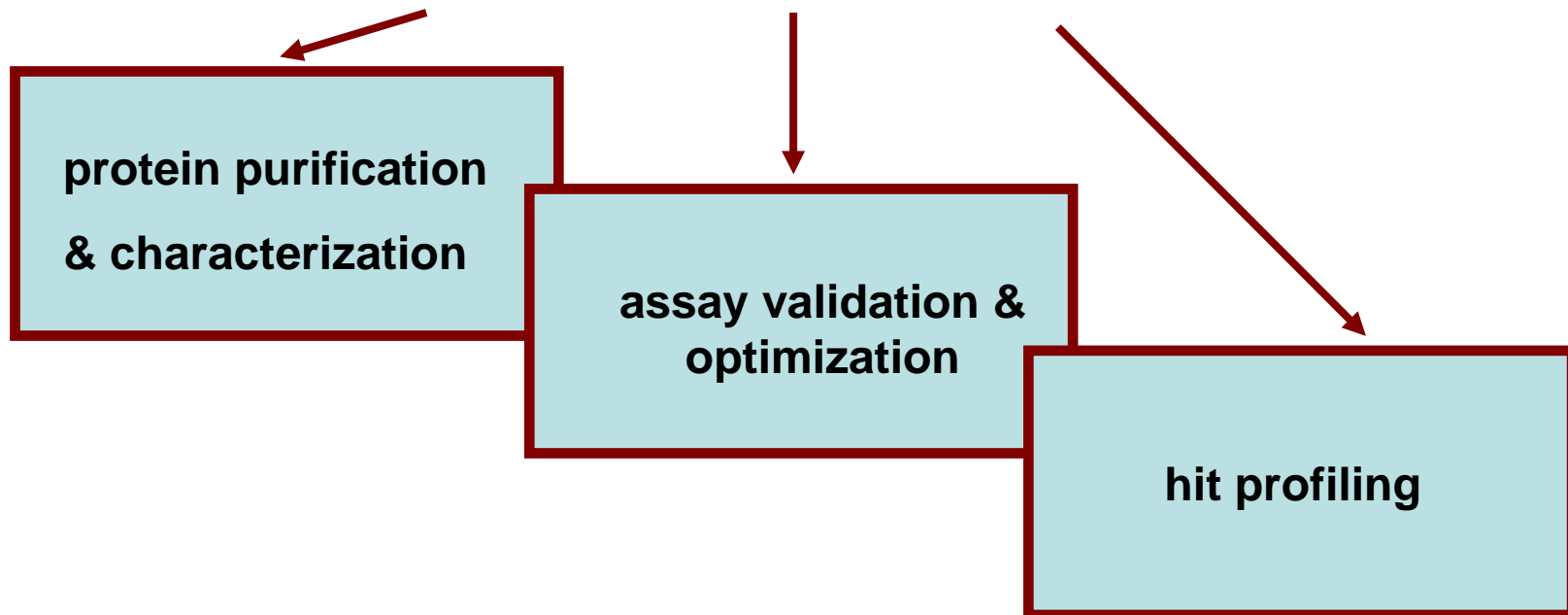
# Biophysical techniques in drug discovery

## Upsides of biophysical techniques:

- ▶ first principle data produced with fewer assumptions and simplifications
- ▶ possibility to cross-correlate experimental findings to assure their reliability
- ▶ better control over experimental parameters and the reacting species ▶ better possibility to eliminate artifacts

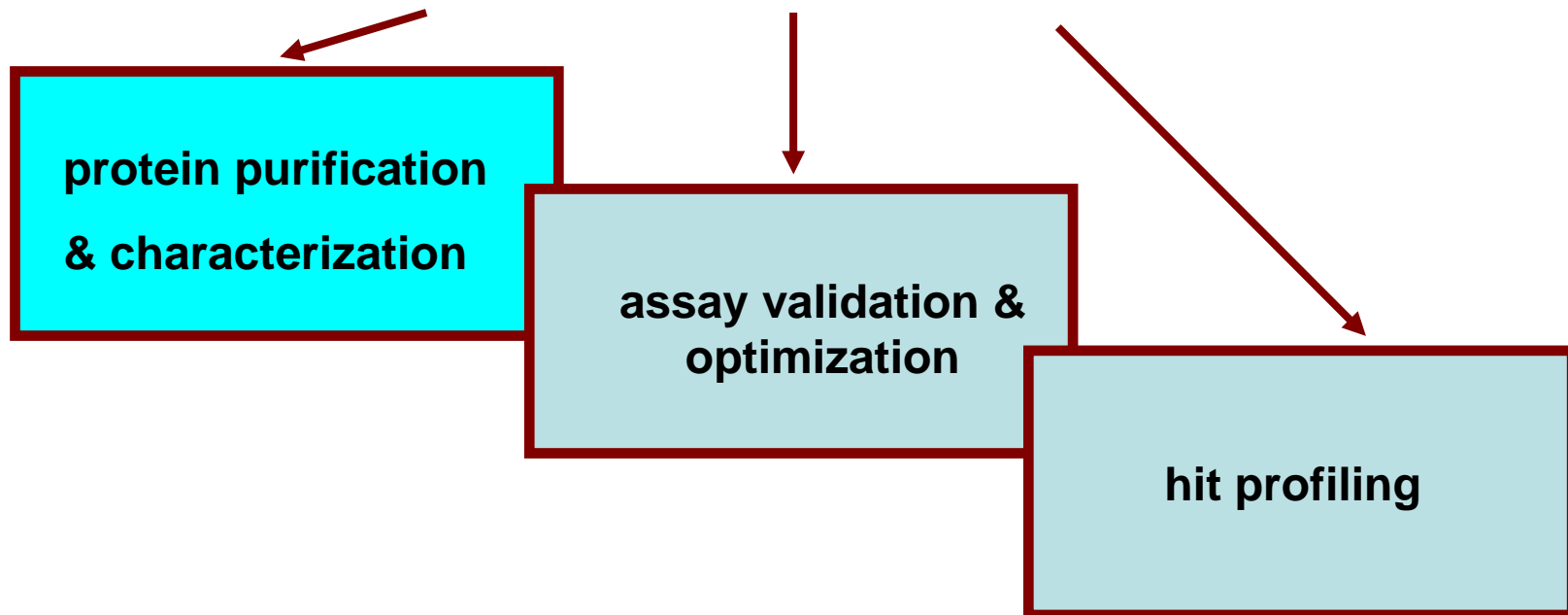
# Biophysical techniques in drug discovery

## Main areas of application



# Biophysical techniques in drug discovery

## Main areas of application



# Biophysical techniques in protein purification and characterization

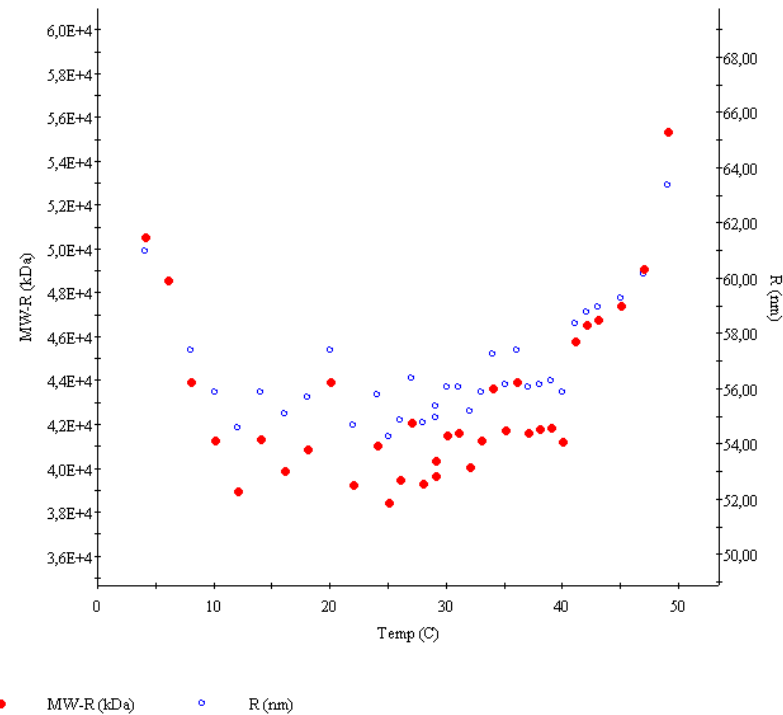
## Protein purification:

- ✓ identification of factors critical to protein stability
- ✓ optimization of purification conditions

## Protein Characterization:

- ✓ homogeneity (posttranslational modifications, presence of fortuitous ligands, extent of self-association and aggregation)
- ✓ oligomerization state
- ✓ thermal stability profile
- ✓ enzymatic activity

# Identification of factors critical to protein stability

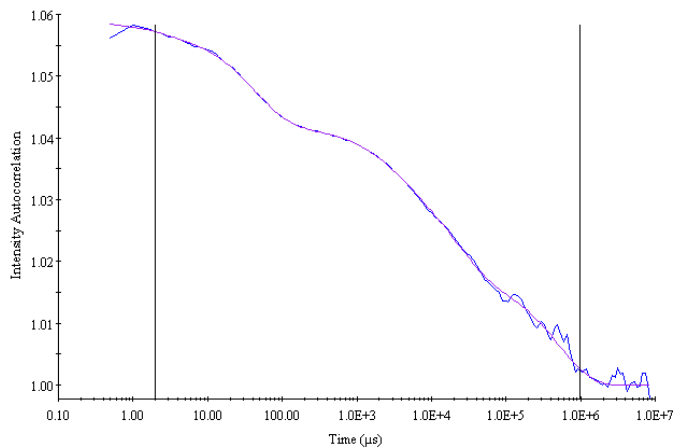


➤ **DLS**: temperature-dependent self-association of protein X.  
Purification at low temperatures should be avoided.

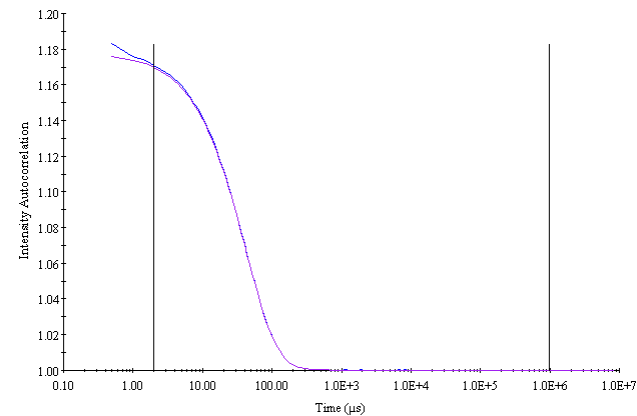
# Identification of factors critical to protein stability

Samples of Protein X taken at different stages of purification process were characterized with biophysical methods.

**Protein X after shear stress against glass surface**



**Protein X after shear stress against plastic surface**

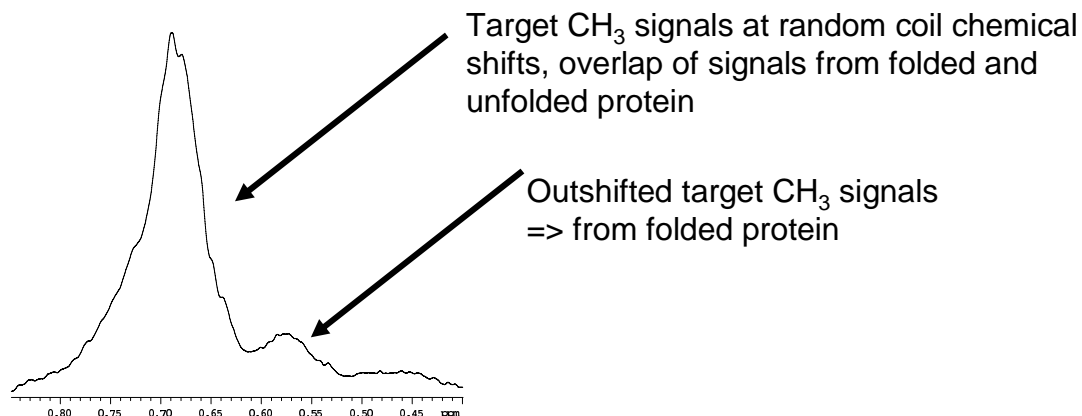


➤ **DLS and DSC**: Protein X is extremely sensitive to shear stress in a contact with glass especially at low pH.

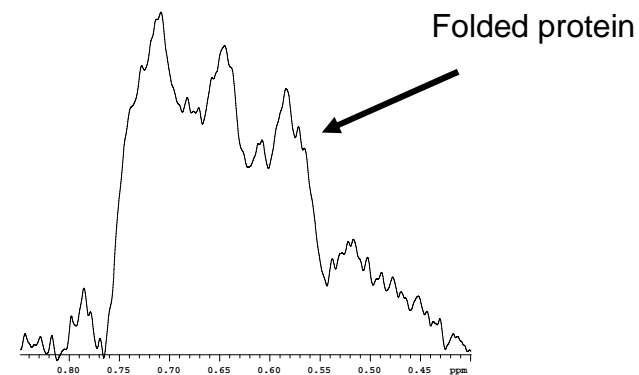
# Quick diagnostic of a protein state: NMR

Methyl region of  $^1\text{H}$  1D spectra of target protein directly after thawing:  
Protein state in two different storage buffers

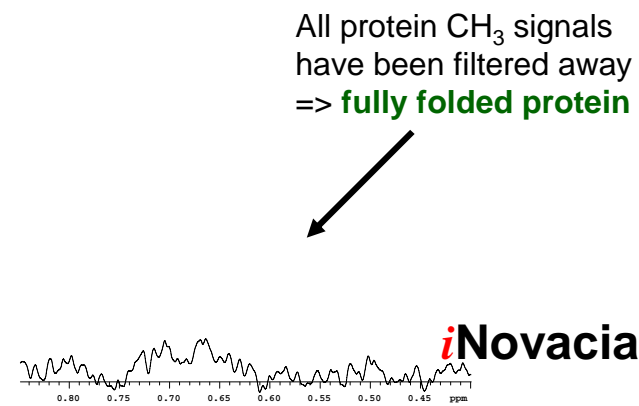
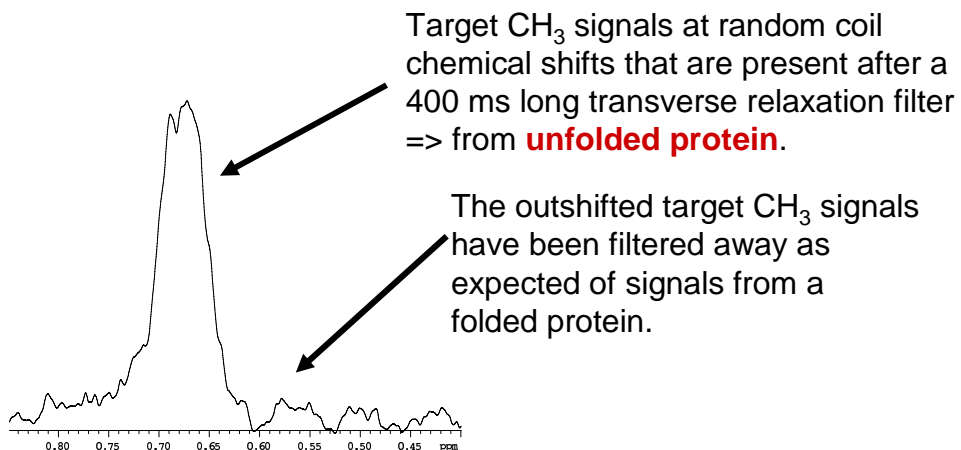
## Phosphate buffer



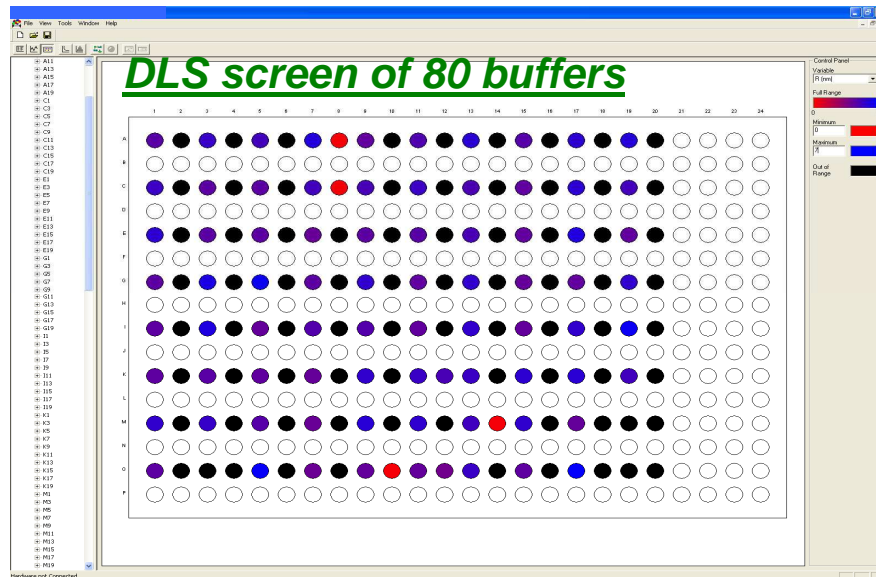
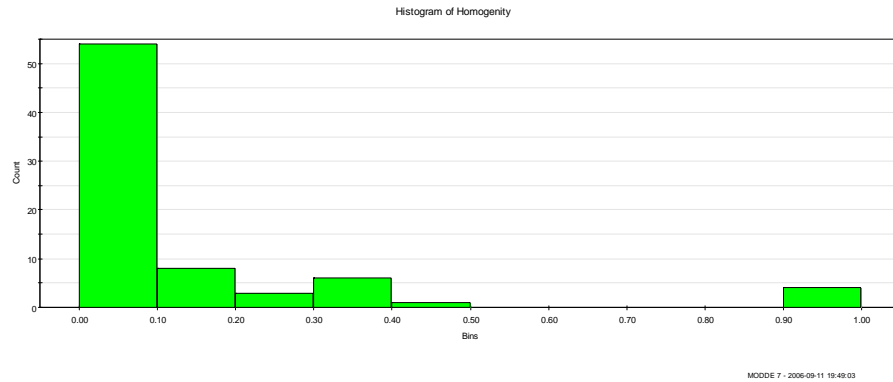
## HEPES buffer



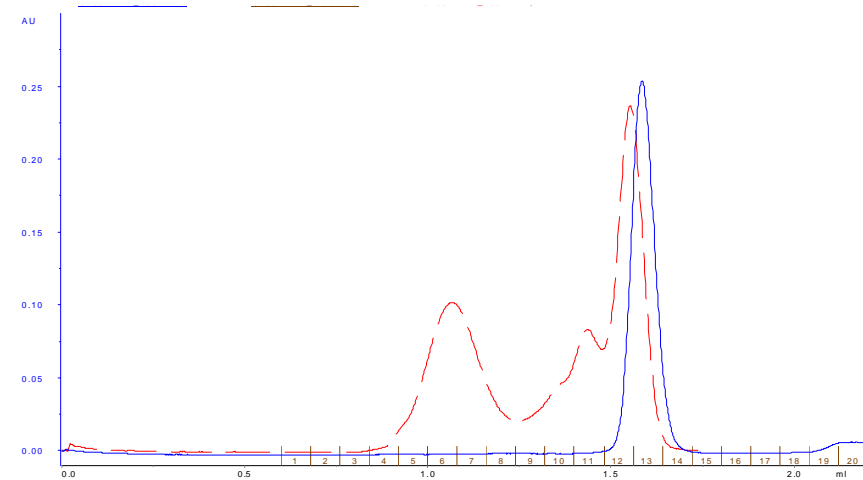
Transverse relaxation filtered  $^1\text{H}$  1D spectrum of target protein (spinlock=400 ms)



# Optimization of purification conditions: buffer optimization screen



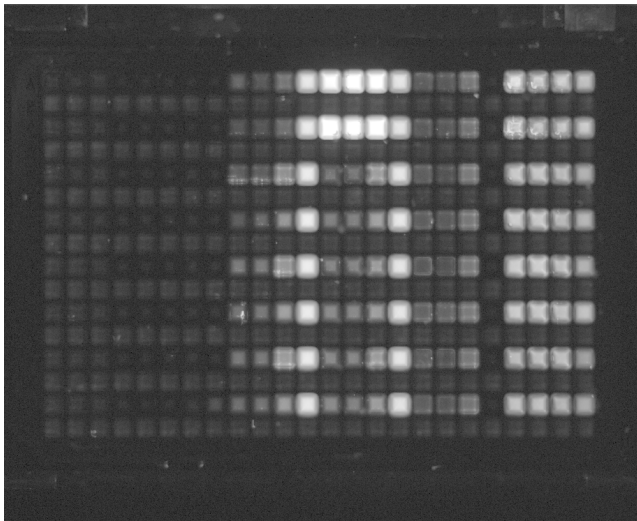
**SEC**



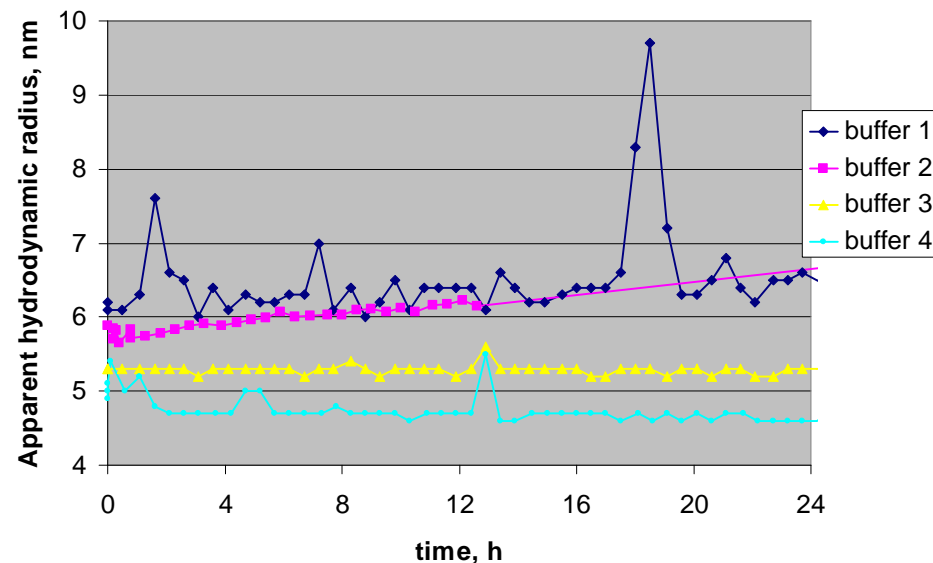
➤ **DLS** helped to identify the buffer conditions optimal for Protein Y homogeneity

# Buffer optimization in stress stability study of Protein XX

Fluorescent intensity measurements: ViewLux

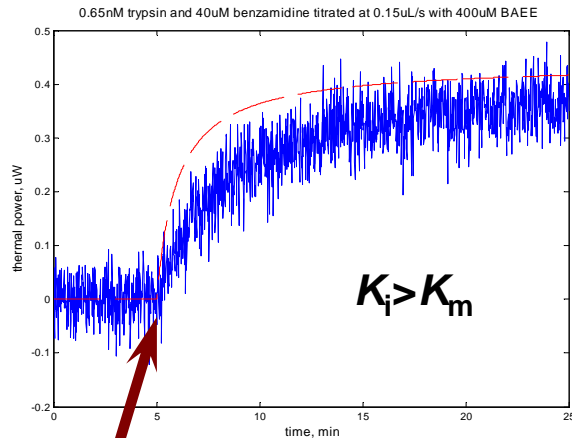


DLS plate reader: Time-dependent homogeneity of Protein XX in the worst and the best buffers



➤ Fluorescence measurements and DLS: conditions resulting in an improved stability of concentrated Protein XX to freeze-thaw cycles were identified

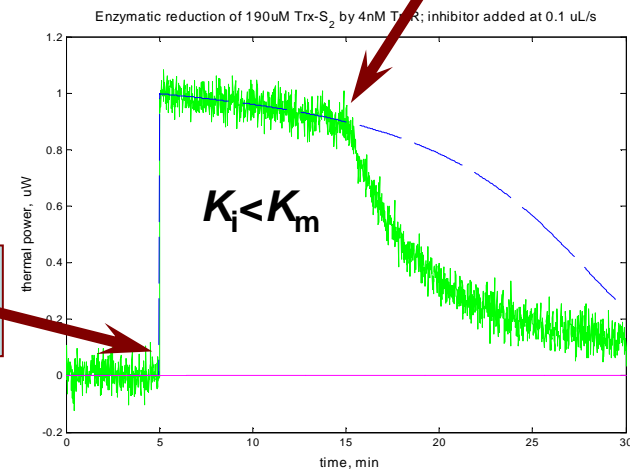
# Characterization of enzyme kinetics with microcalorimetry



Continuous injection of a substrate

	cITC	Ref
$K_{cat}$ , $s^{-1}$	19.0	16-22
$K_m$ , $\mu M$	2.6	4
$K_i$ , $\mu M$	21	16
$-\Delta H$ , kJ/mol	50	48

Injection of an enzyme



Continuous injection of an inhibitor

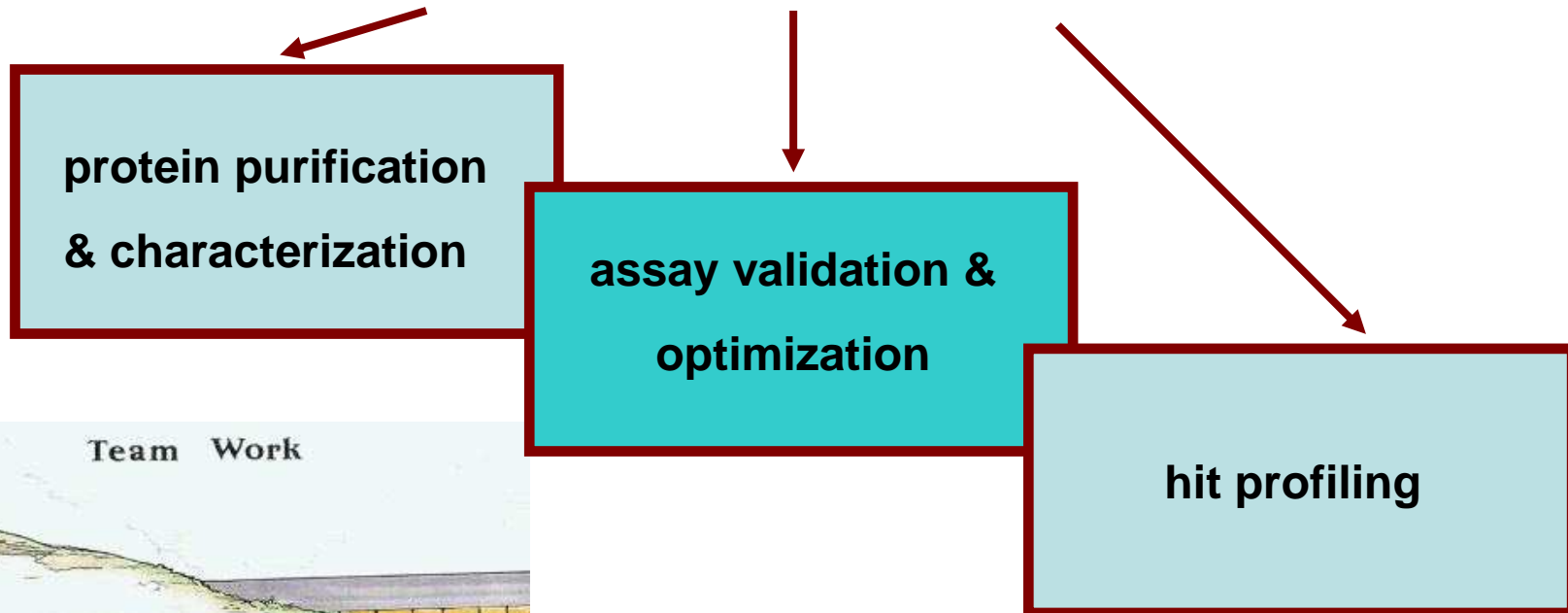
	cITC	Ref
$k_{cat}$ , $s^{-1}$	45	55
$K_m$ , $\mu M$	17	26
$K_i$ , nM	110	60
$\Delta H$ , kJ/mol	7	-

➤ **IMC**: generic assay with no need for:

- optically clear samples
- chemical modification of the reacting species
- a system of coupled reactions

# Biophysical techniques in drug discovery

## Main areas of application



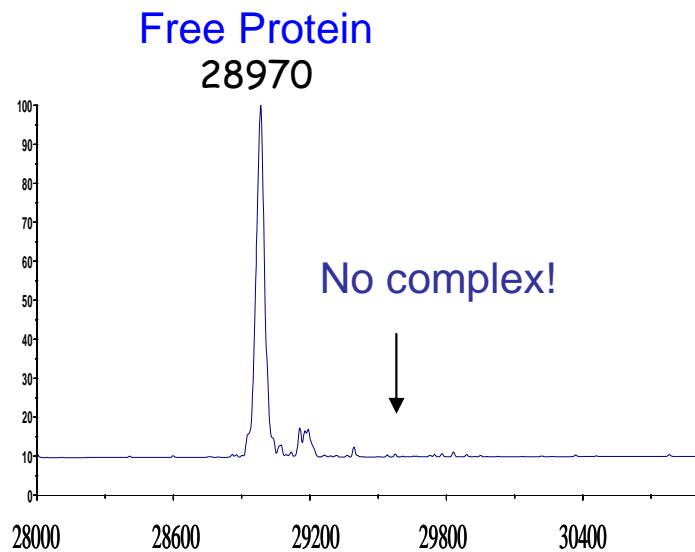
# Biophysical techniques in assay development

**Why are the results from assay and other techniques not reproducible? Why the protein activity is low?**

- ✓ Protein chemical and physical inhomogeneity (posttranslational modifications, presence of unknown ligands, extent of aggregation)
- ✓ Thermal lability
- ✓ Protein in oligomerization state with low activity
- ✓ Protein instability in the assay or during storage
- ✓ Poor compatibility with buffer component/s
- ✓ Not optimal protein construct
- ✓ Unexpected behavior of a reference compound or other reagents

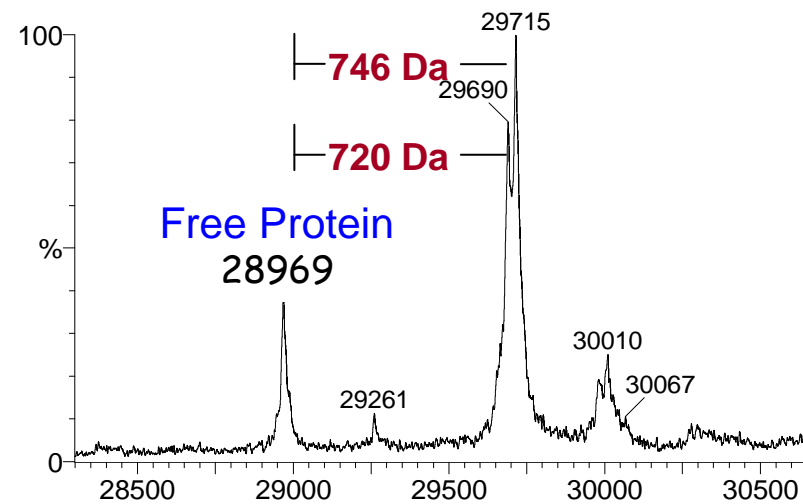
# Protein inhomogeneities: Presence of endogenous/fortuitous ligands

## MALDI-TOF MS on denatured protein:



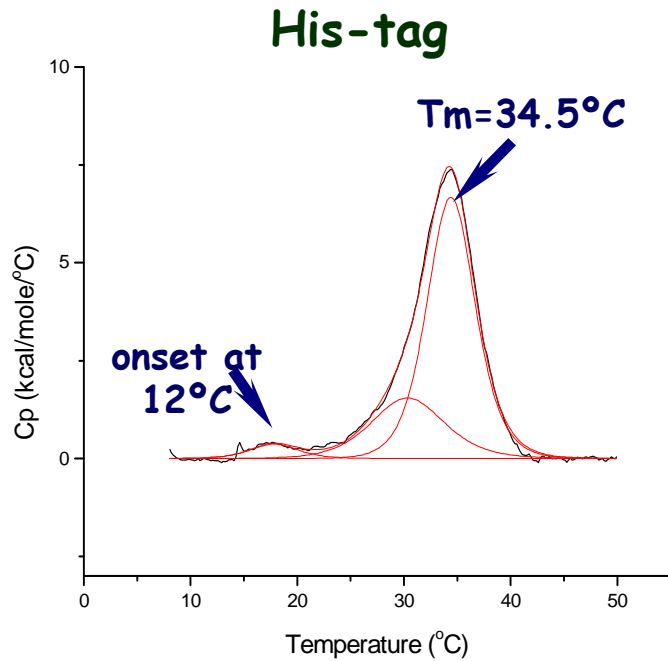
**Denatured protein:**  
Only the free protein is  
observed at 28.9 kDa.

## Nondenaturing ESI-MS:



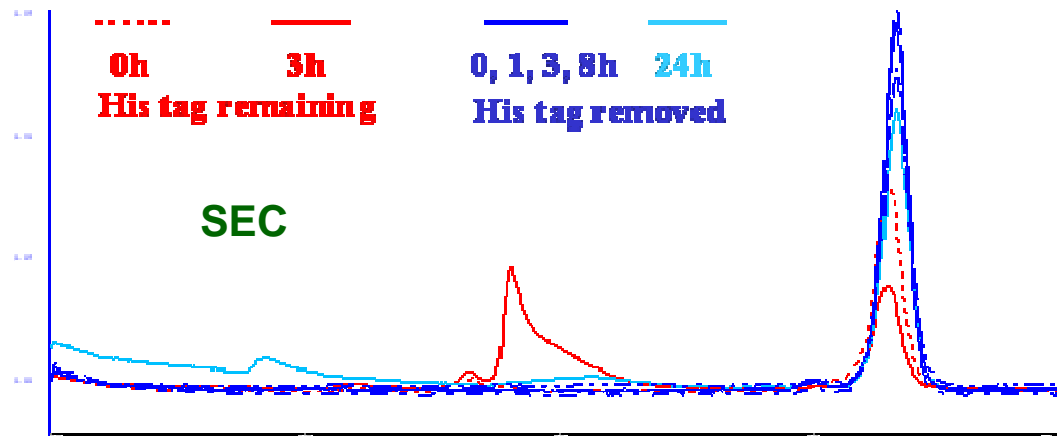
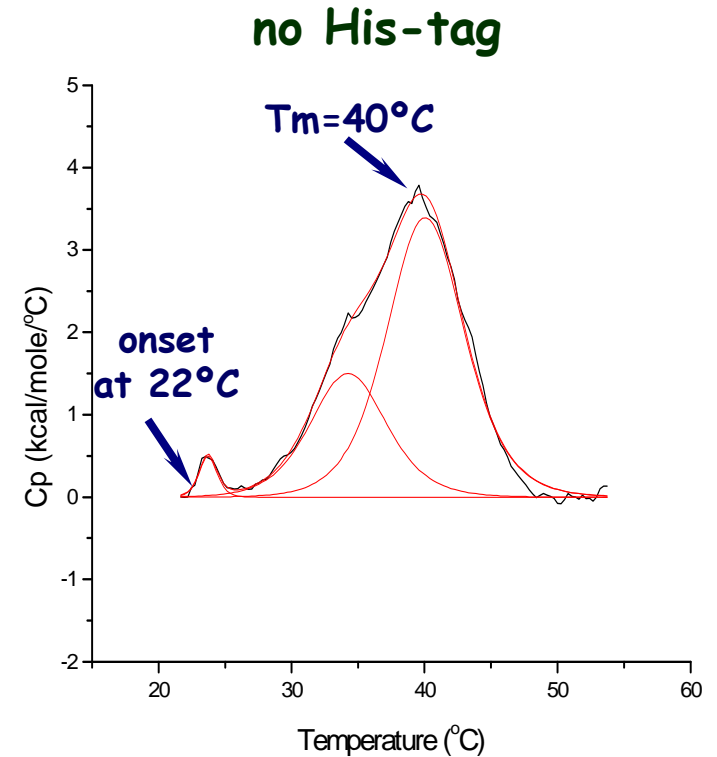
**Native protein:**  
Two protein-ligand complexes  
were observed and identified

# Protein thermal lability



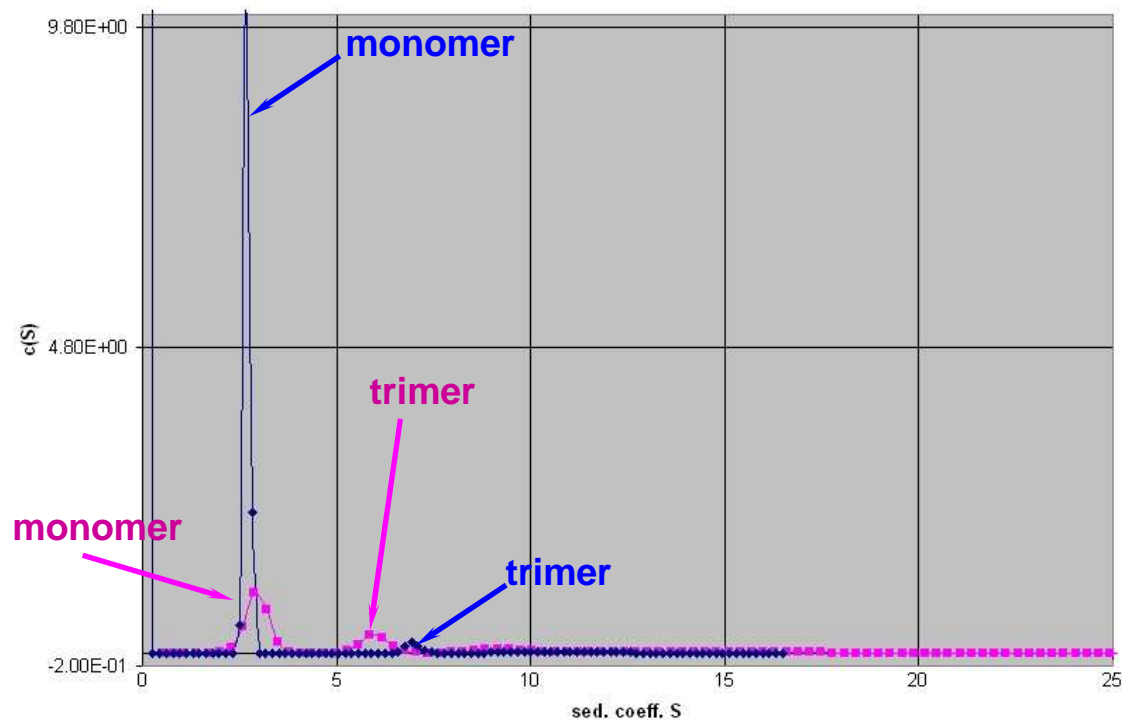
➤ **DSC:** removal of a His-tag resulted in a significant increase of the thermal stability.

➤ **DSC:** 10°C upward shift of the low-temperature transition stabilizes the protein kinetically.



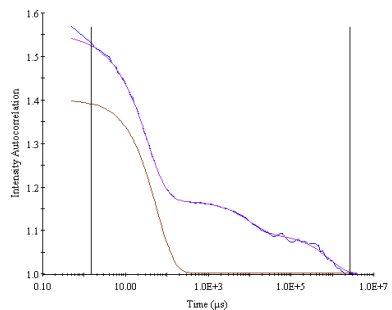
➤ **SEC:** protein homogeneity and stability to aggregation has dramatically increased

# Protein activity related to oligomerization state

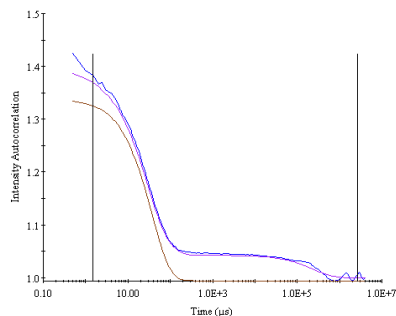


➤ **AUC**: One of the two protein batches contains larger fraction of trimers. Trimer content was well correlated with the protein activity.

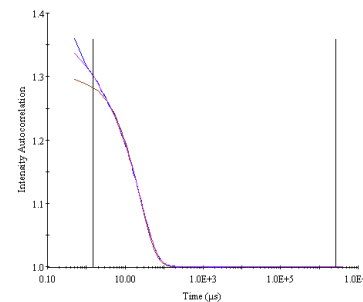
# Choice of a protein batch for assay development & screening



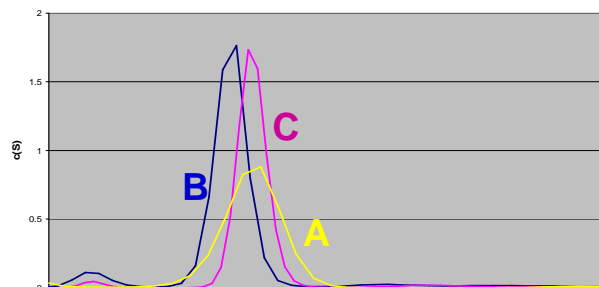
Batch A



Batch B

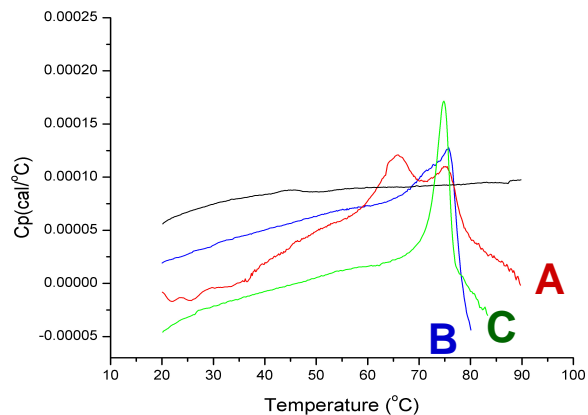


Batch C



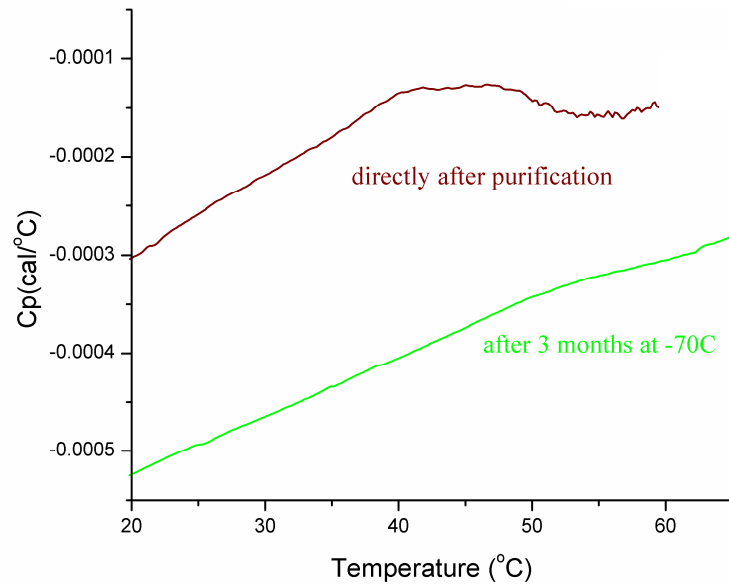
## DLS, AUC and DSC:

- Batch A: structurally/compositionally heterogeneous, thermally labile.
- Batch B: structural/compositional homogeneity of the main specie has increased but this batch contains significant fraction of fragments and aggregates.
- Batch C: homogenous and most stable.

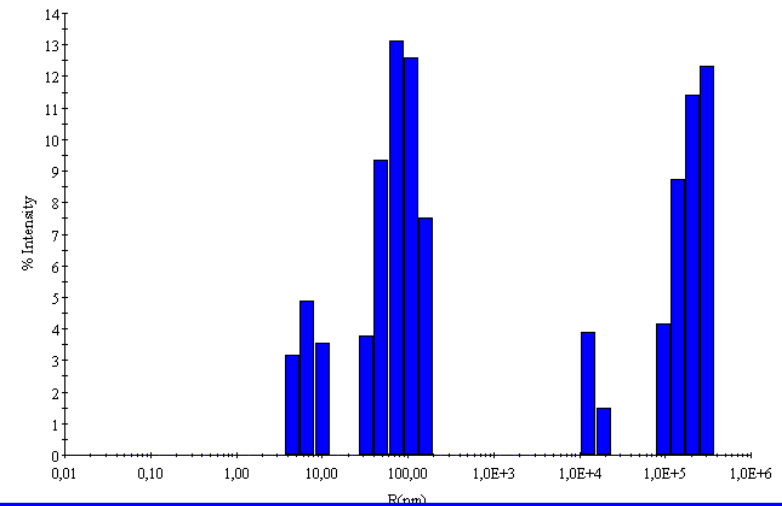


# Protein instability upon storage

## DSC



## DLS



Item	R (nm)	%Pd	MW-R (kDa)	%Mass
Peak 1	7.2	34.5	343	99.2
Peak 2	90.0	45.1	125869	0.2

➤ **DSC**: loss of tertiary structure after storage at -70°C.

➤ **DLS**: After 3 months storage at -70°C essentially all of the protein is aggregated.

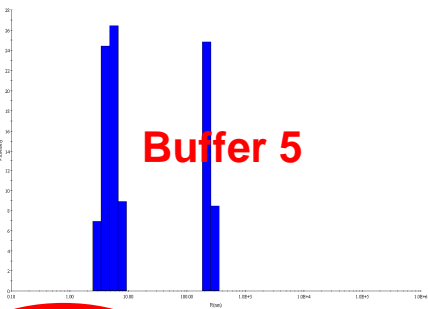
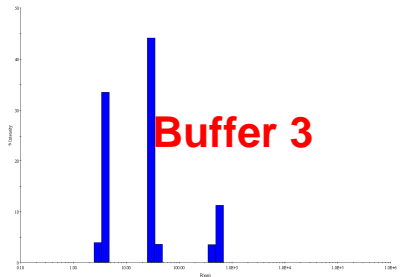
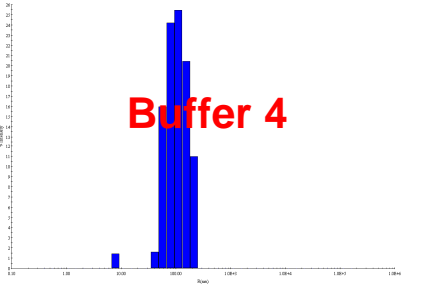
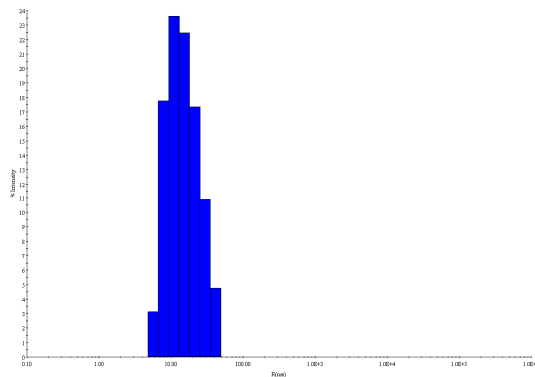
# Protein instability in assay due to reversible aggregate formation induced by freezing/thawing

Item	R (nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	3.0	10.7	43	70.9	99.8
Peak 2	18.9	15.4	3345	29.1	0.2

Item	R (nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	7.9	3.1	419	1.4	86.2
Peak 2	114.3	42.8	219692	98.6	13.8



Protein S in original buffer

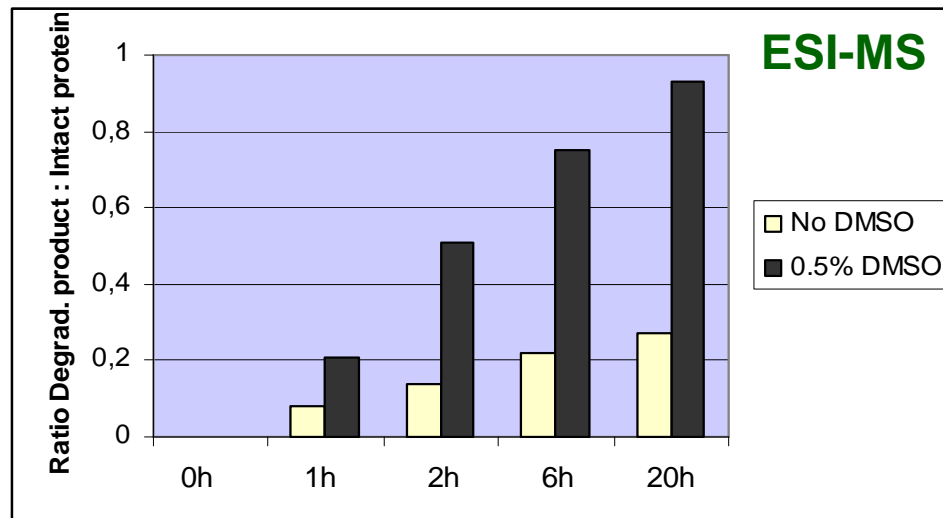
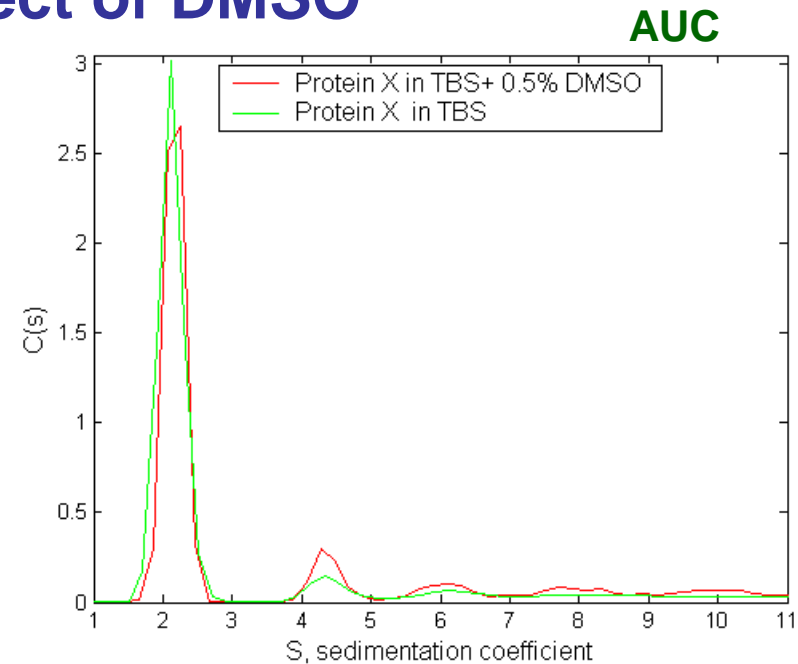
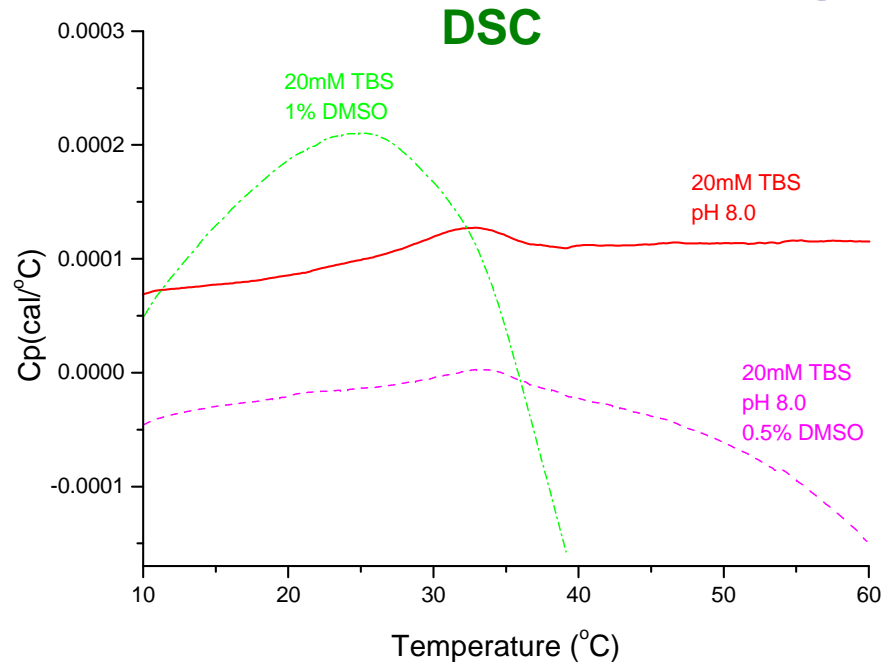


Item	R (nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	4.0	8.9	84	37.5	99.7
Peak 2	30.6	10.0	10078	47.7	0.3
Peak 3	546.1	12.9	8549230	14.7	0.0

Item	R (nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	5.1	28.1	154	66.7	99.9
Peak 2	239.3	15.5	1239910	33.3	0.1

➤ **DLS:** buffer-dependent dissociation of protein aggregates formed during freeze-thaw. Buffer favorable for efficient dissociation was identified.

# Poor compatability with a component of assay buffer: destabilizing effect of DMSO

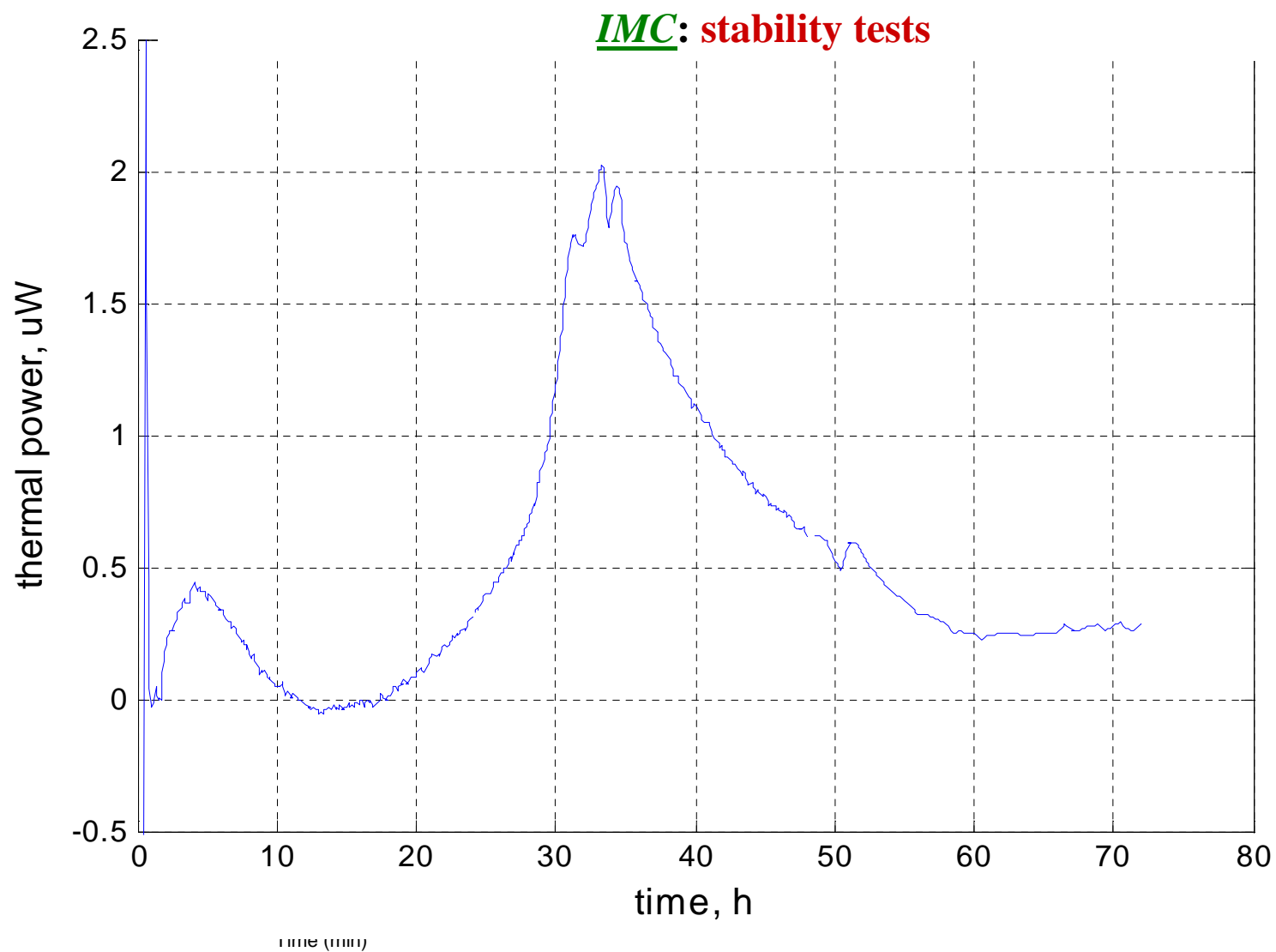


## DSC, AUC and ESI-MS:

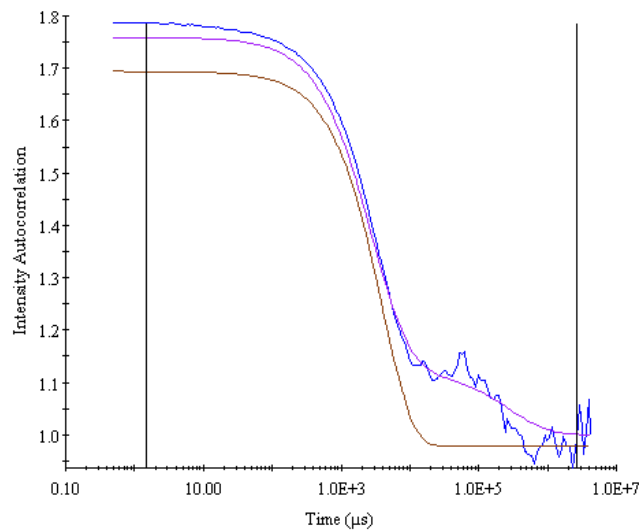
➤ At 0.5% DMSO (approx. 60 mM) large fraction of the protein has lost tertiary structure, protein unfolds already at room temperature.

➤ Rate of degradation increases.

# Irreproducible assay results due to intrinsic instability of reference compound

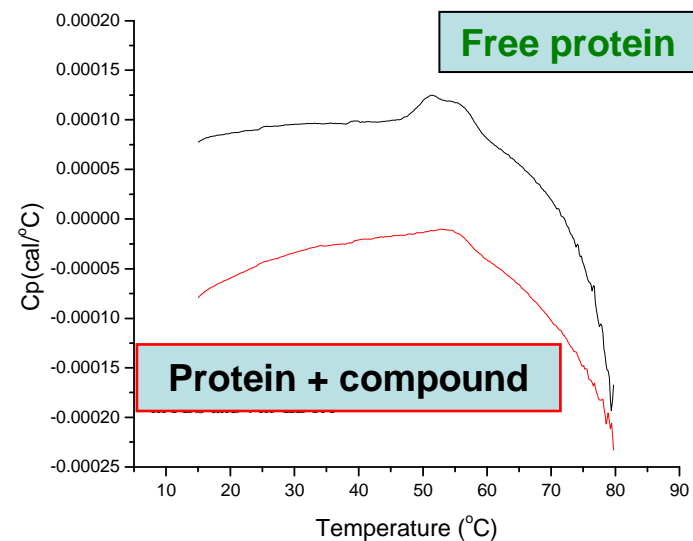


# Irreproducible assay results due to poor solubility of a reference compound



## DLS:

➤ reference compound is not completely soluble at 10 mM in DMSO and at 50  $\mu\text{M}$  concentration in the assay buffer.

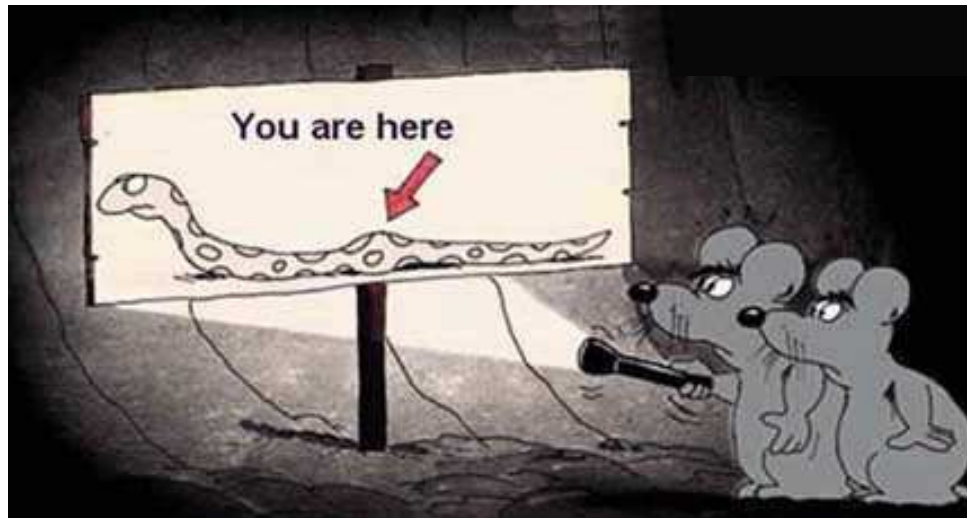


## DSC:

➤ reference compound known as a reversible binder destabilizes Protein X

# *Can biophysical techniques make a difference?*

- especially valuable in the projects dependent on in-house protein production
- help to early detect and adequately address protein stability issues
- give valuable insights into the nature of irreproducible assay results and low protein activity



# Acknowledgements

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