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Advances in the integrated measurement of higher order protein structure and size

Dynamic Light Scattering and Raman Spectroscopy combined to provide insights into sample denaturation and aggregation

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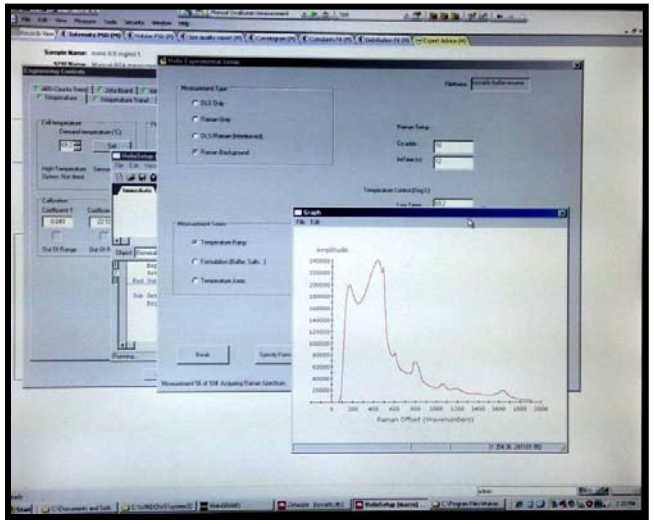
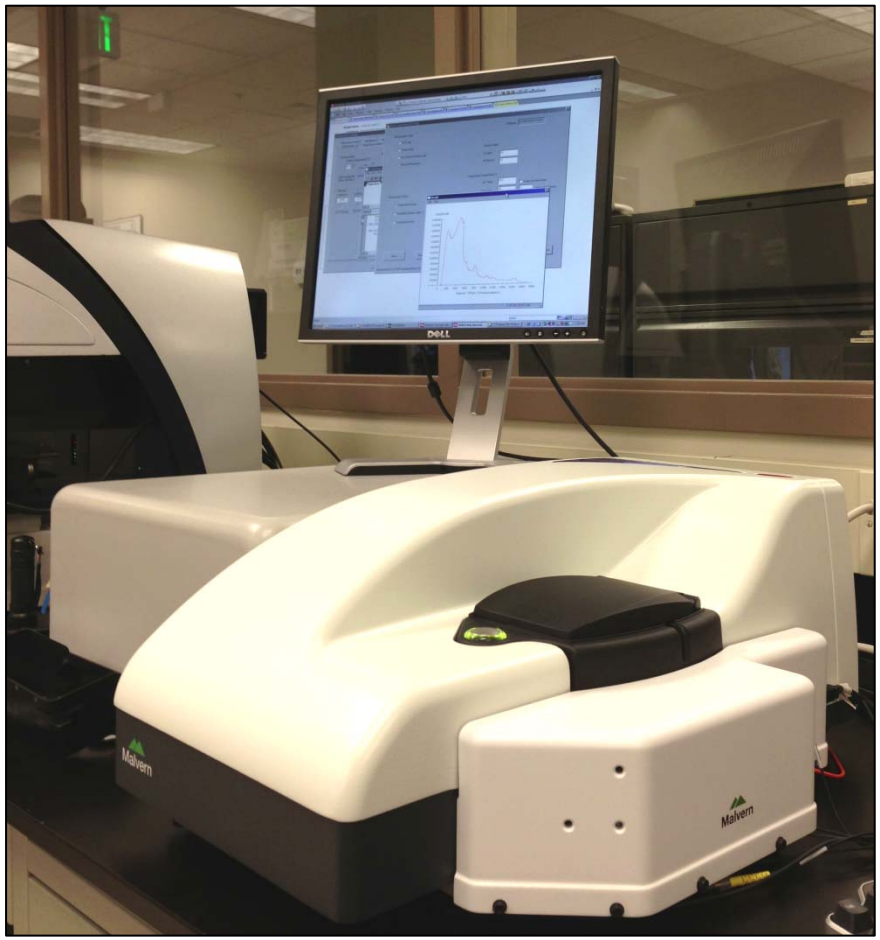
BDI

- Malvern Instruments' Bioscience Development Initiative (BDI) is a company established to accelerate innovation, development and promotion of new technologies, products and capabilities to address unmet measurement needs in the biosciences markets.
- Neil Lewis was previously CEO of Spectral Dimensions (NIR spectroscopy) and now is the manager of this BDI team

DLS & Raman combined





Sample:

- 45 µl minimum volume
- Temperature: 0 to 90°C, +/- 0.1 °C

RamanRxn1™ Specifications:

- 785 nm laser excitation
- Frequency: 150 cm^{-1} to 1850 cm^{-1}
- Resolution: 4 cm^{-1}
- Concentration: up to 50 mg/ml

DLS Zetasizer NanoZS Specifications:

- 633 nm laser excitation
- 0.3nm – 10 microns
- Concentration: up to 50 mg/ml
- Forward & back scatter (2 angles)

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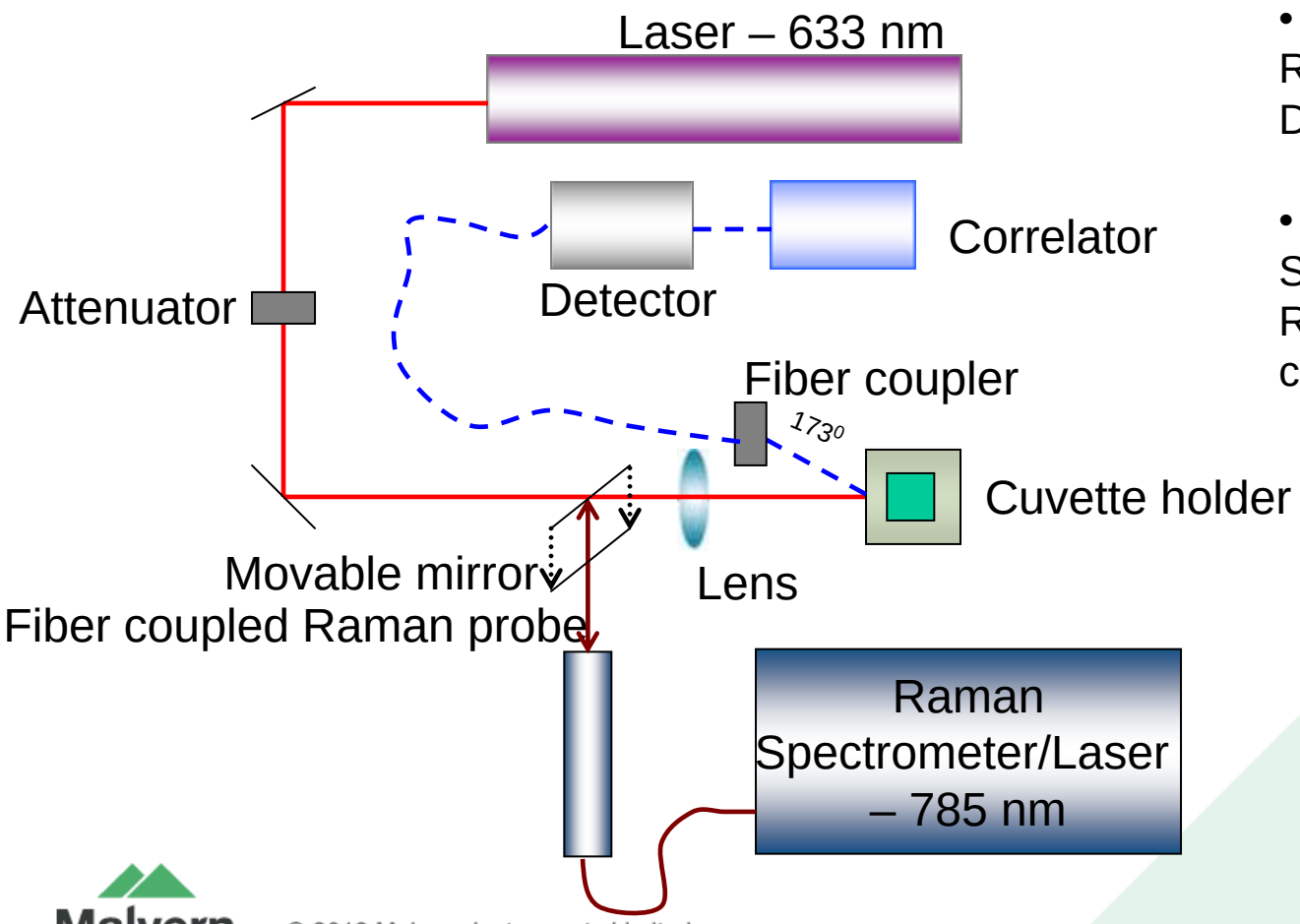
Schematic of Experimental Setup



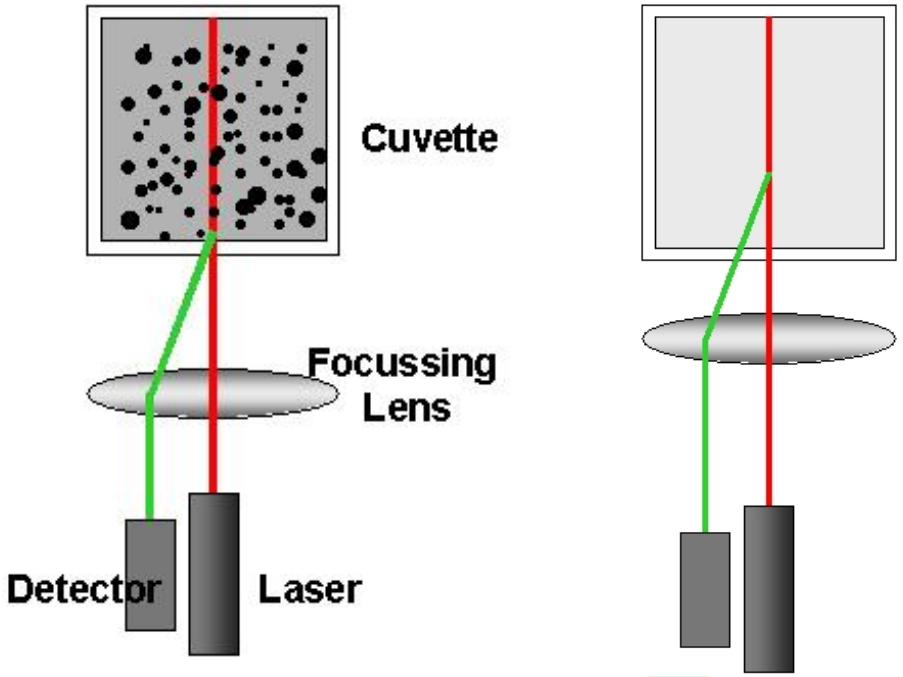
Data is collected in an interleaved fashion:

- At each temperature: Raman spectra and then DLS data are collected.
- Temperature changed: System equilibrates, next Raman and DLS data are collected.

Optics in NIBS (Non-Invasive Back Scattering)

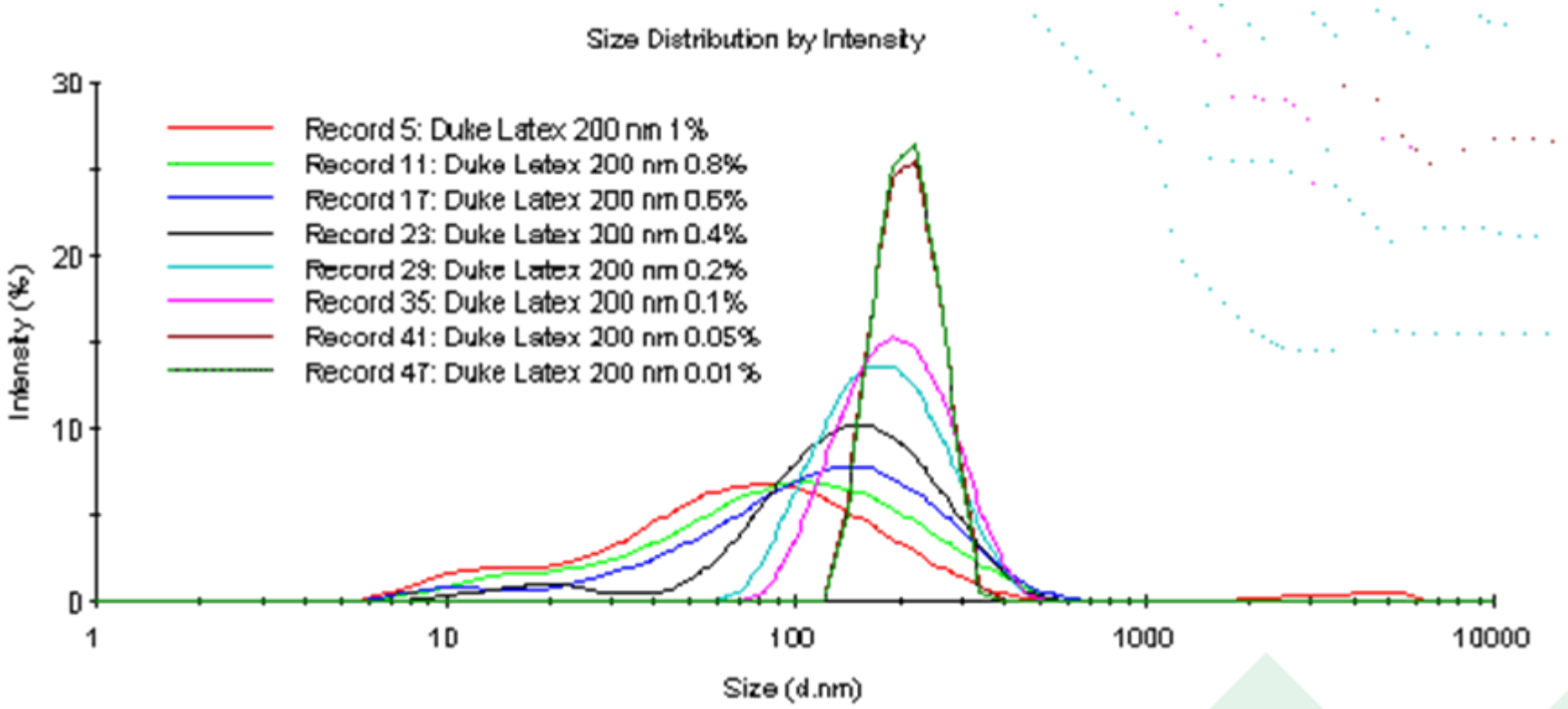
- NIBS Patent for high concentration

  - **Optics are not in contact with sample**
  - **Measurement angle = 173°**



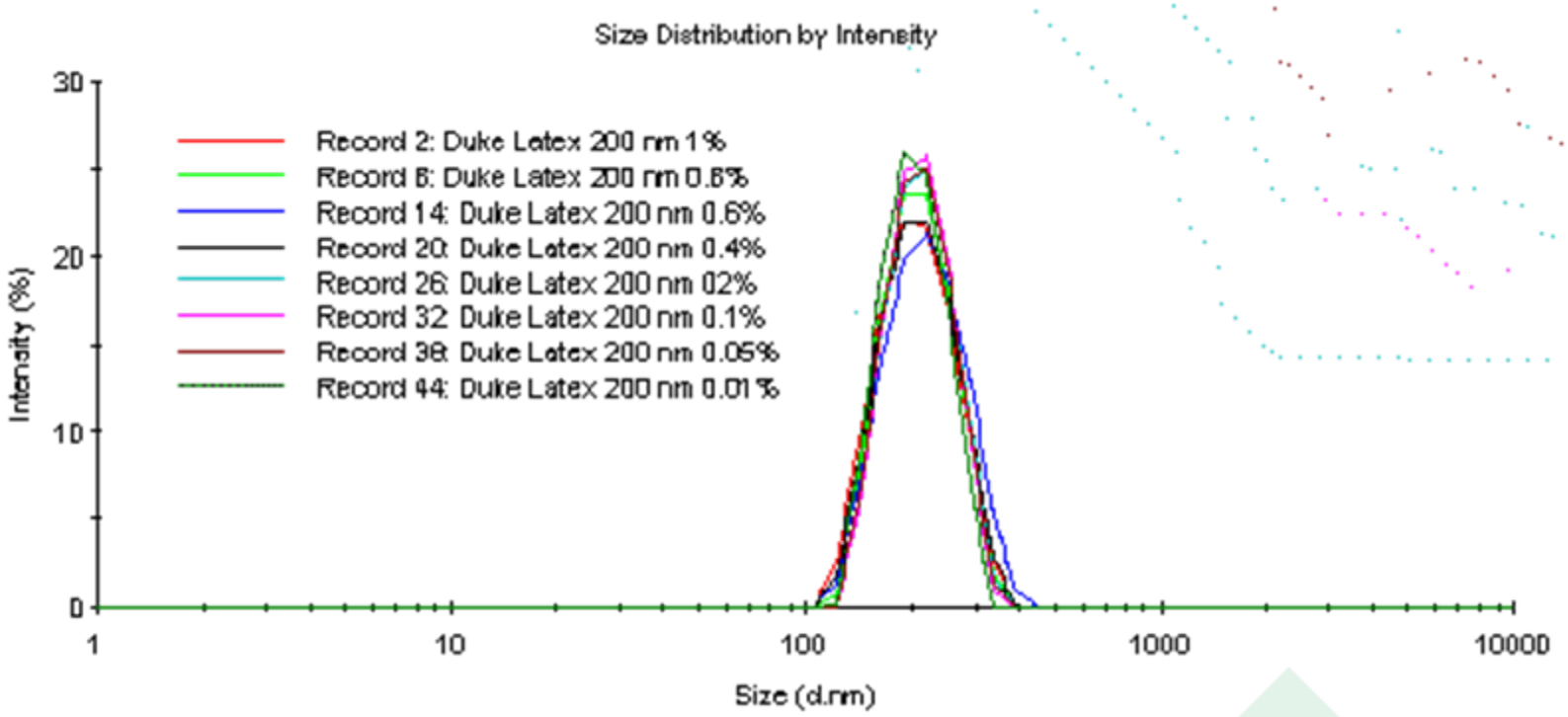
Measurement zone moves to reduce multiple scattering and collective diffusion

Effect of multiple scattering in the result



- Latex 200nm measured without NIBS **in the center of a cuvette**. Depending on increasing concentration, results become wider and even very polydisperse or bimodal

Avoiding multiple scattering with NIBS



Latex 200nm measured with NIBS and **on the side of cuvette**.
With increasing concentration, result remains unchanged

Raman spectroscopy for secondary and tertiary structure of protein

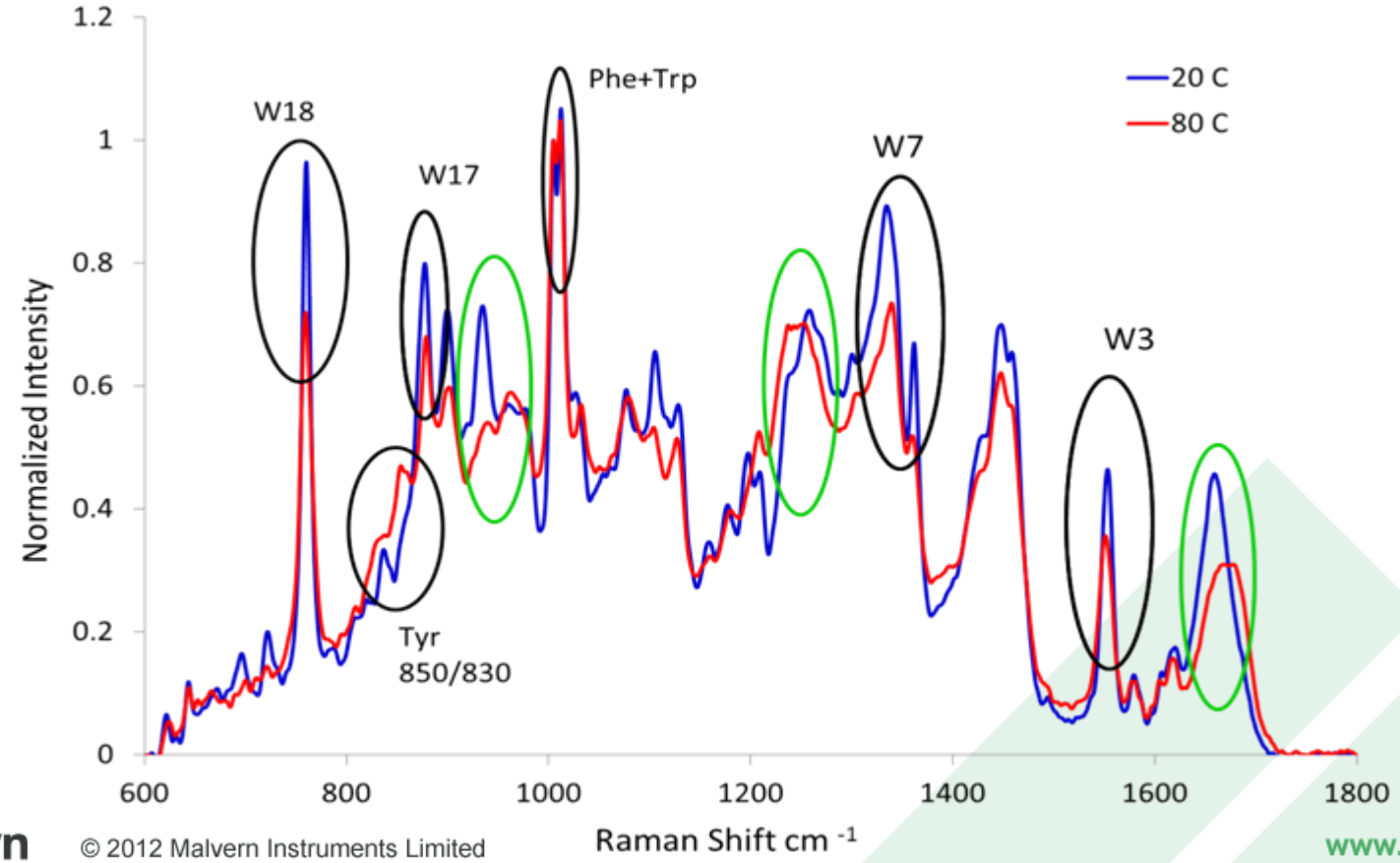
- Raman spectroscopy derives protein secondary structure (amide I and III) and tertiary structure markers (aromatic side chains, disulfide bond, hydrogen bonding, local hydrophobicity).
  - Bonding interactions between side chains
  - Folds, bends and loops in protein chain – fragments of the same chain bonded together
  - Hydrogen bonding
  - Disulfide bonds (covalent)
  - Hydrophobic interactions – non-polar amino acids point to the interior
- The structural changes can be monitored by the determination of spectral peak position, shape, and/or intensity and is label free.
- Raman make these structural determinations at formulation concentrations, 50 mg/mL or even greater!

Structural Information derived from Raman Spectra of Protein

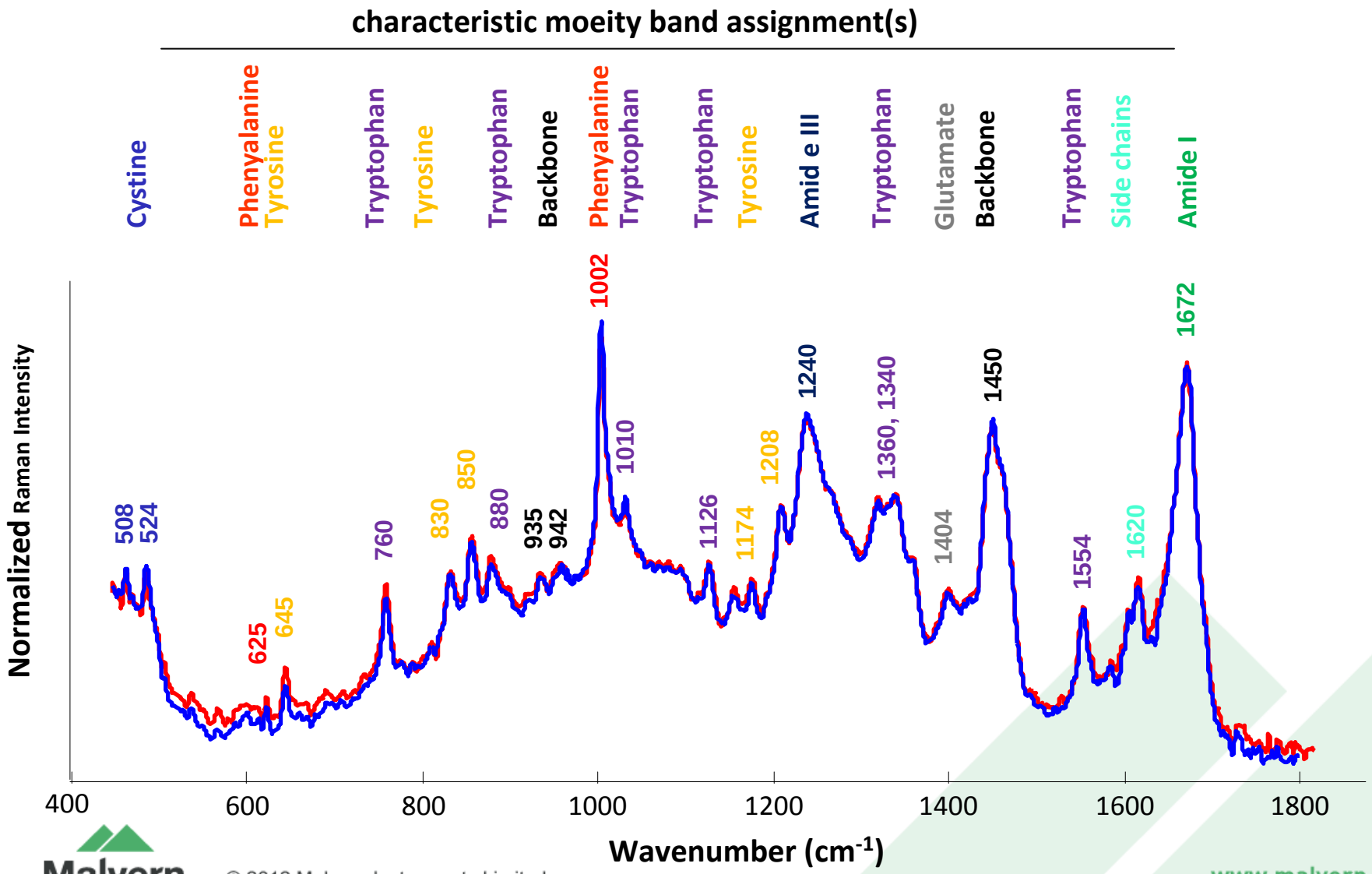
	Band position (cm-	Description	Reference
Secondary structure	1680	Amide I - beta turn	Wen, 2007
	1665	Amide I - beta sheet	Anderson, 2003
	1653	Amide I - alpha helix	Anderson, 2003
	1450	CH2,CH3 deformation	
	1320	CH stretch	
	1298	Amide III - N-H bend (O=C-N-H)	
	1248	Amide III - N-H bend (O=C-N-H)	
	930-950	N-C_α-C skeletal stretch - alpha helix	Wen, 2007; Levin, 2006
	1209	sym ring stretch	Wen 2007
Tyrosine	1174		Liang 2006, Wen 2007
	856	phenol hydroxyl group doublet (829)	Liang 2006 , Wen 2007
	829	phenol hydroxyl group doublet (856)	Liang 2006, Wen 2007
	645	C-C twist, C-S stretch (gauche)	Levin, 2006
	1767	hydrophobic/hydrophililic environment	Wen 2007
Tryptophan	1575		Wen 2007
	1554	dihedral angle - indole ring to peptide bond plane	Levin, 2006
	1460		Wen 2007
	1360	fermi doublet indole ring (1340)	Liang 2006, Wen 2007
	1340	fermi doublet indole ring (1360)	Liang 2006, Wen 2007
	1238		Wen 2007
	1126		Liang 2006, Wen 2007
	1010		Wen 2007
	880	hydrogen bonding - 871 strong, 883 no H-bonding	Liang 2006, wen 2007
	760	hydrophobic/hydrophilic environment	Tuma, 2001, Liang 2006
Side Chains	1615	C=C stretch - Tyrosine, Tryptophan, Phenyl	Levin, 2006
	1605	C=C stretch - Tyrosine, Phenyl	Barman, 2012
	1404	COO- stretch - Glutamate	Barth, 2002
Phenylalanine	1584		Barman, 2012; Notingher 2007
	1205		Wen 2007
	1030	C-H ring deformation	Levin, 2006
	1002	aromatic ring breathing	Levin, 2006
	625	C-C twist	Levin, 2006
Cystine	704	C-S stretch (trans)	Wen, 2007
	666	C-S stretch (gauche)	Tu, 2003 and Levin
	544	S-S stretch (trans-gauche-trans)	Levin, 2006
	524	S-S stretch (gauche-gauche-trans)	Levin, 2006
	508	S-S stretch (gauche-gauche-gauche)	Levin, 2006

Lysozyme submitted to thermal stress

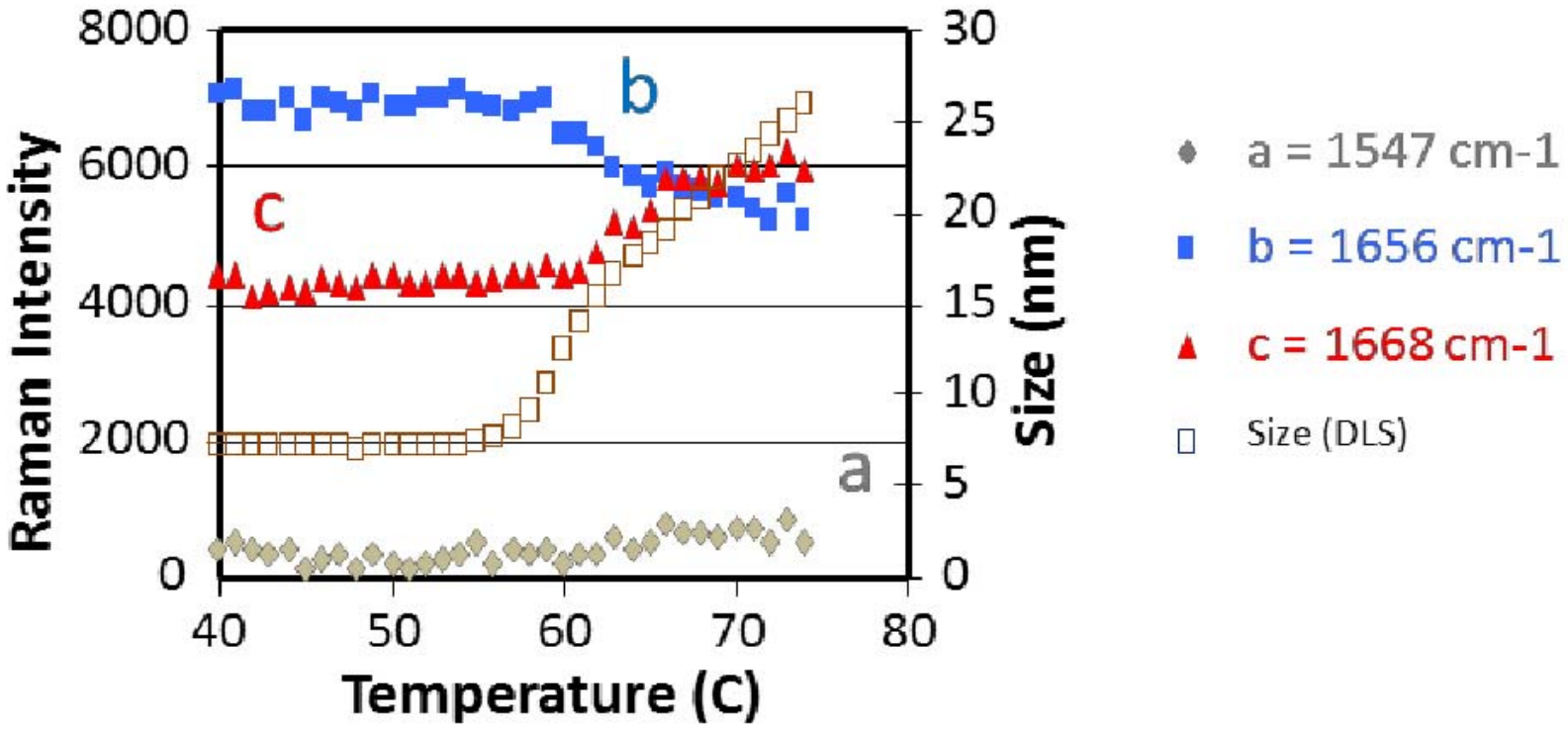
- Raman spectra of lysozyme (pH 7.4) before and after heat treatment **blue line=20°C, red line =80°C**
- The secondary structure (**green circles**) of the lysozyme changes when the temperature increases. These changes are illustrated with peak shifts and/or intensity changes.
- The tertiary structural markers indicate changes in the aqueous environments of the aromatic side chains (**black circles**), changes in the hydrogen bonding of the side chains, and changes in the dihedral bonding angles. Lysozyme is undergoing structural changes while exposed to thermal stress



Raman Spectra of mAb's

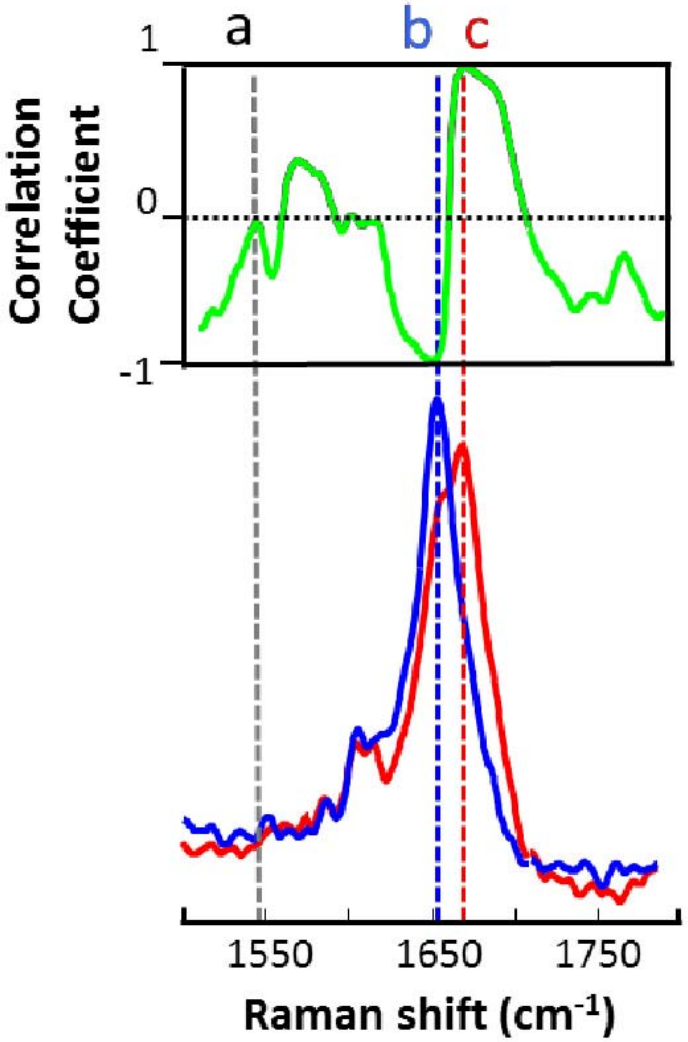


Correlation between DLS and Raman spectra for BSA vs temperature



Analysis of DLS and Raman data for a 10 mg/ml BSA solution.
Trend plot comparing change in Raman intensity (at 1547, 1656 and 1668 cm^{-1}) with size as function of temperature.

Correlation between DLS and Raman spectra



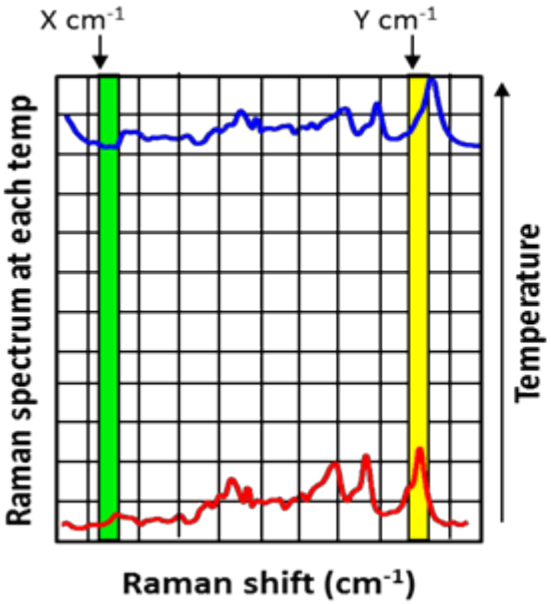
Correlation between Raman Amide I frequency region and hydrodynamic radius is shown in upper graph and Raman Amide I band positions are shown in lower graph.

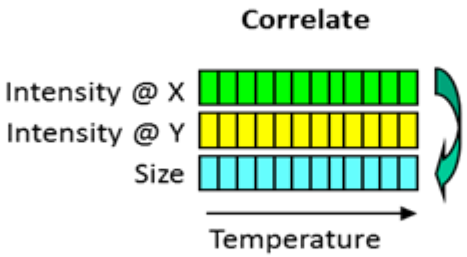
Point b (1656 cm^{-1}) has a negative correlation with size, while point c (1668 cm^{-1}) has a positive correlation with size (upper graph).

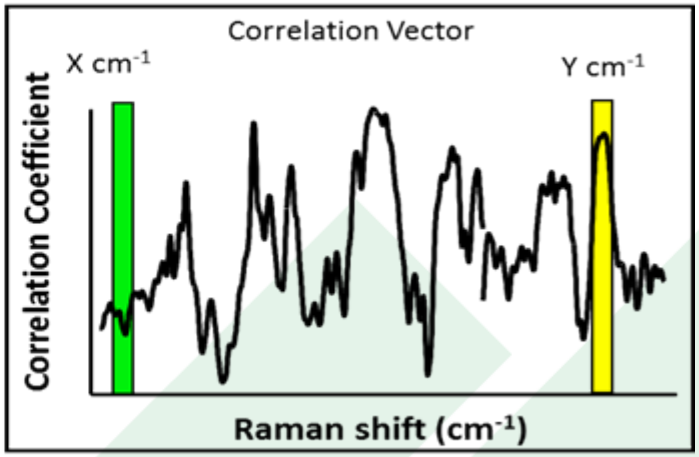
We will calculate correlation vector at each frequency and it is possible to correlate a protein physical property (size) with a chemical change (shape/structure)

Multimodal analytical techniques

- For each Raman frequency on the x axis, a vector can be extracted that tracks the change in Raman intensity at that frequency as a function of the change in temperature (y axis). Then we correlate each of these vectors (one for each Raman frequency) with the change in size obtained from the DLS measurement.
- The result is a correlation vector. The y-axis is the strength of the correlation, and the x-axis is in Raman shift

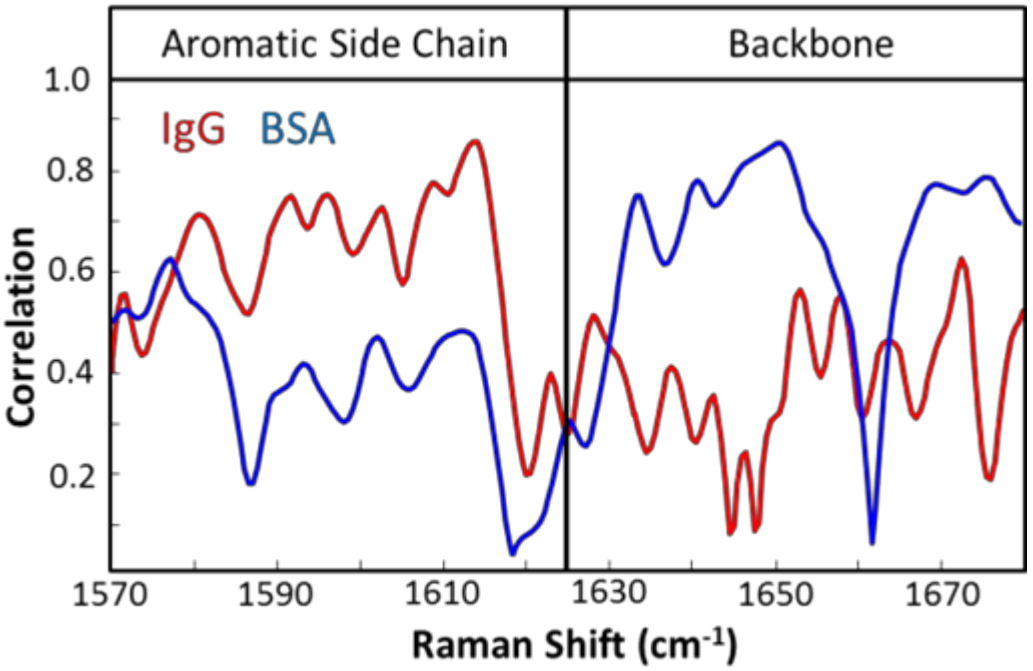






Analysis results with BSA and IgG

- The method described before is applied to IgG and BSA samples (10 mg/ml) that were prepared in pH 7 phosphate buffer. DLS and Raman data were collected between 20 and 74 °C.



For BSA, the correlation vector indicates that the aggregation process is driven primarily by an unfolding of the protein backbone.

For IgG the correlation vector shows that aggregation reflects change in aromatic side chains or tertiary structure, rather than change in the protein backbone or secondary structure.

Analysis of BSA just below melting temperature (Tm)

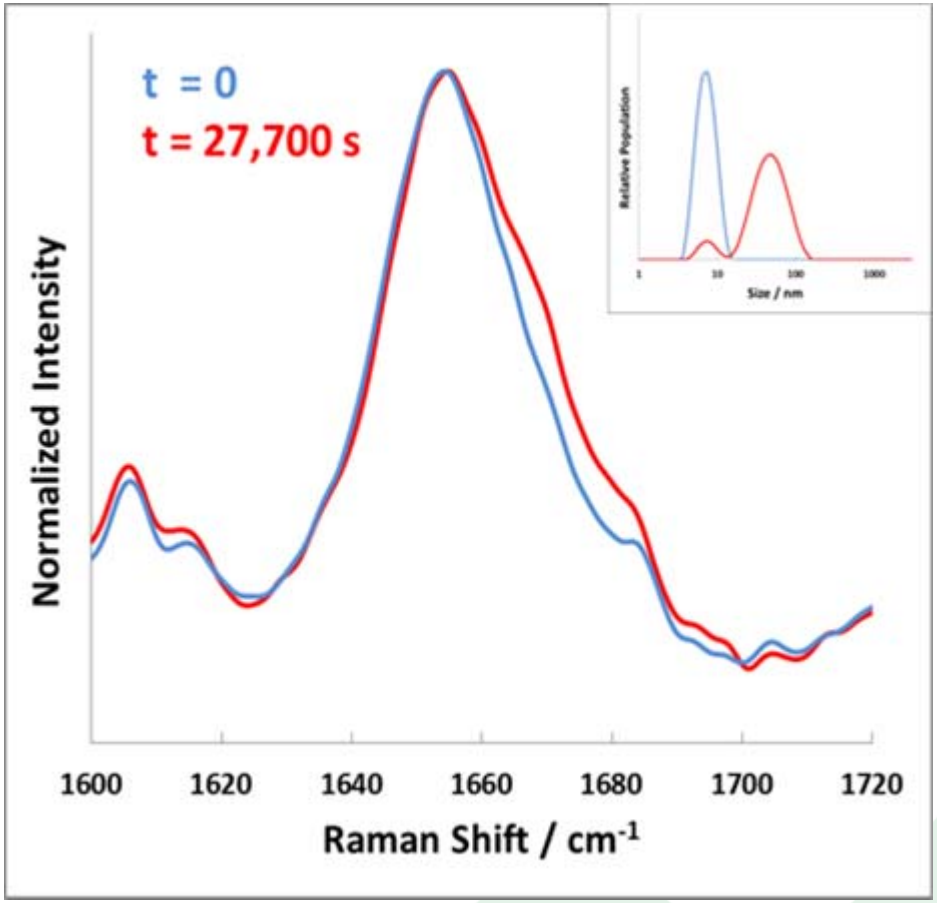
Sample: BSA in citrate buffer at 50 mg/ml concentration, at pH 7.1 at 60°C.

The transition temperature (T_m) was determined for each sample.

Then samples were incubated just below their T_m where unfolding is kinetically slow.

Incubation time t = 0 (blue) and

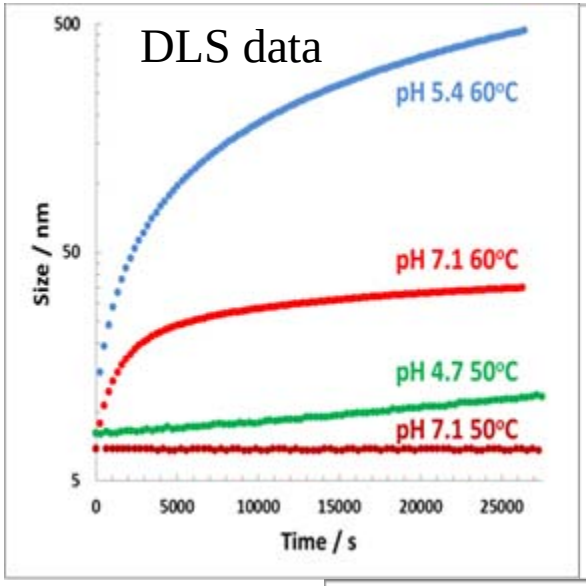
Incubation time t = 7.8 hours (red)

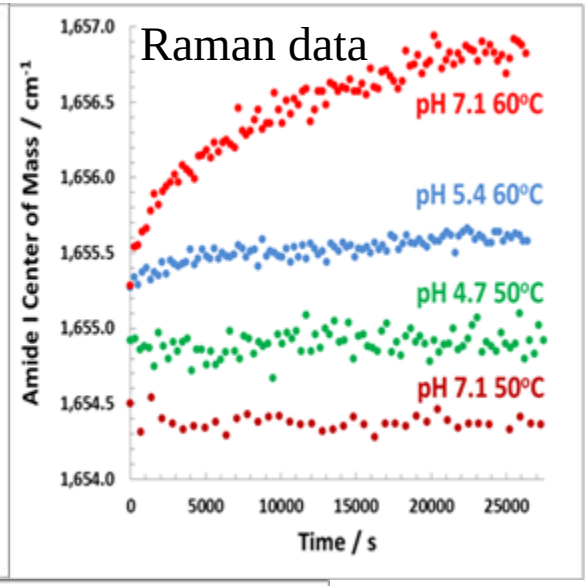


DLS results (inset) shows aggregates between 10 and 100nm.
Amide I band shows a change of shape on higher frequencies

BSA at pH 7,1

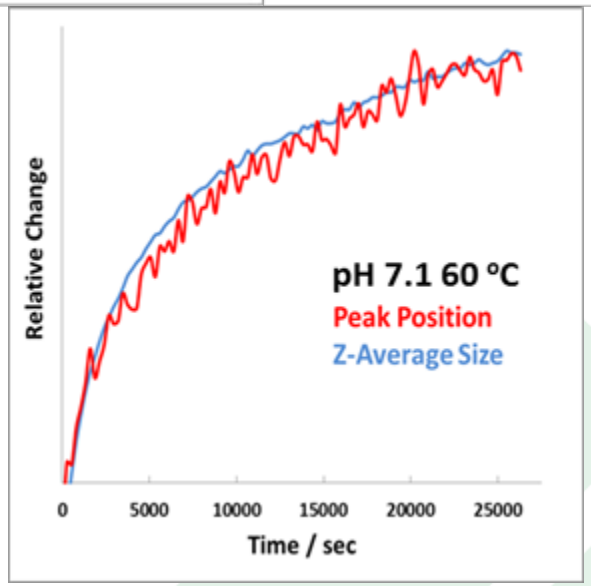
There is significant pH-dependence in the behavior of both BSA unfolding and aggregation.



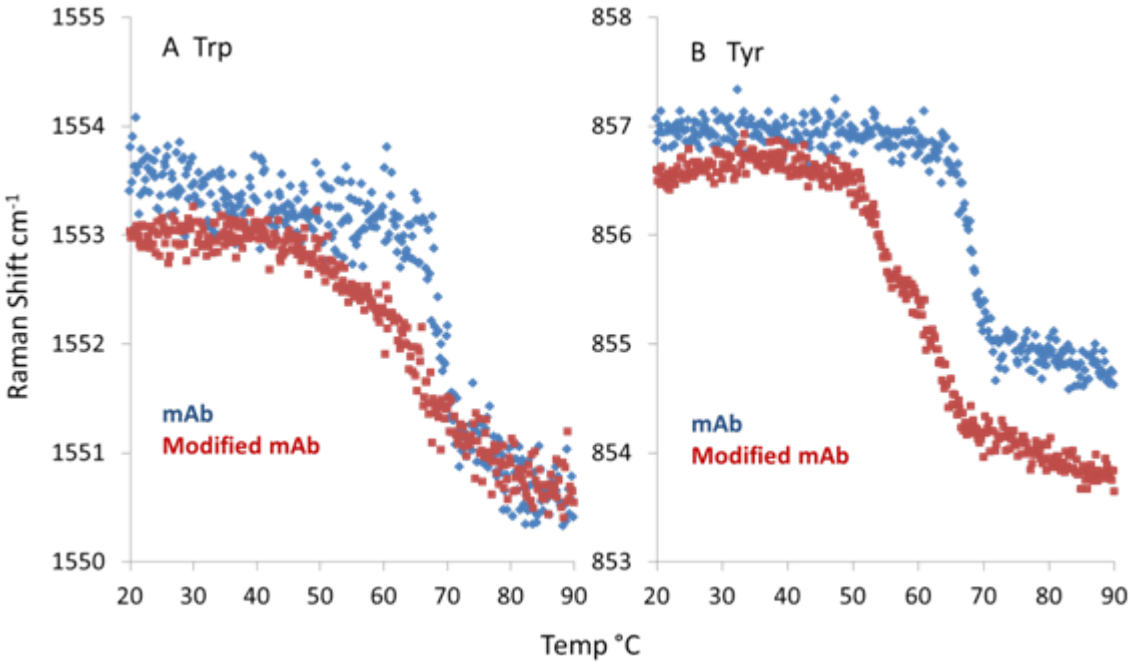


The time-dependence of the amide I band and the *Z*-average size demonstrates that the structural and size changes are well correlated.

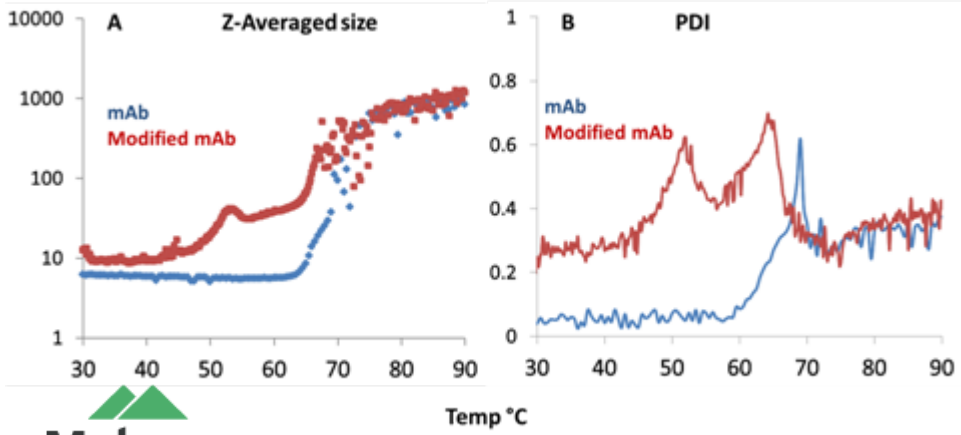
The structural unfolding of the protein results in a larger BSA monomer (up to 35 nm), but little aggregation of the BSA occurs.



Thermal Ramping on mAb and Modified mAb



- For the Tyrosine band, mAb shows a single abrupt transition between 60 to 70 °C
- Modified mAb shows a lower onset temperature with a double transition at ~50 and 65 °C
- 50°C = oligomerization
- 65°C = severe aggregation
- Same variation in Tryptophan band



- PDI trend for mAb and mmAb is consistent with Tyrosine band.
- For mAb Z average increases at ~60°C
- For mmAb Z average inceases at ~50 and 62°C
- Oligomerization event first
- Oligomers forming large aggregates at the higher T transition

Summary

- Label free technique
- No need to dilute
- Other possible applications:
  - Influence of ligand on thermal shift (Tm)
  - Analysis of links between proteins
  - Analysis of links between proteins and ligands
  - Analysis of links between DNA and drug vectors

Thank you for your attention.

References

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