Multi-Detection SEC / SLS-SEC

Stefan Cairns PhD, Product Technical Specialist
Stefan.cairns@malvernpanalytical.com

OMNISEC

MULTI-DETECTOR SIZE EXCLUSION CHROMATOGRAPHY SYSTEM

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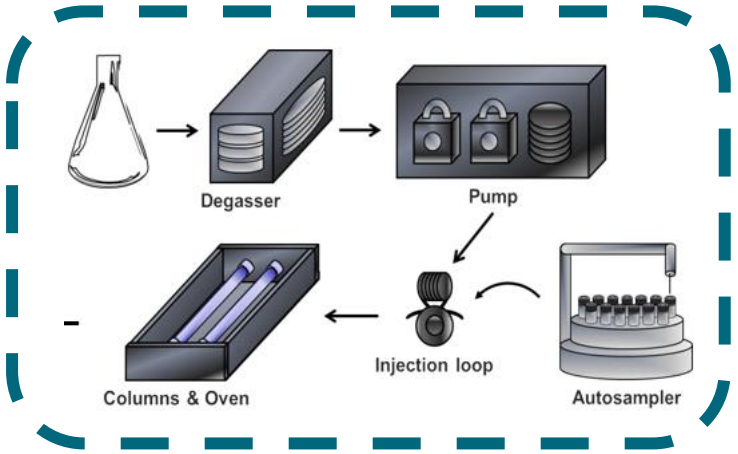

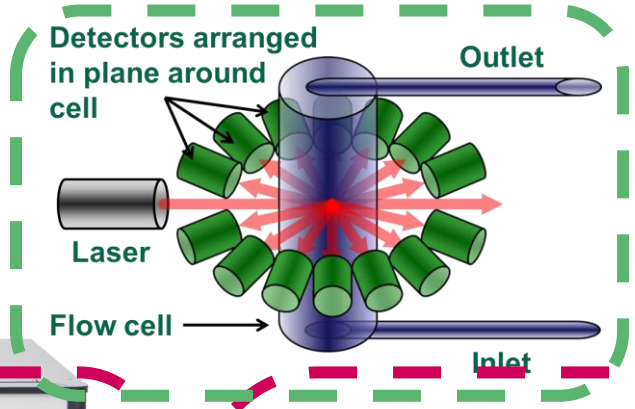

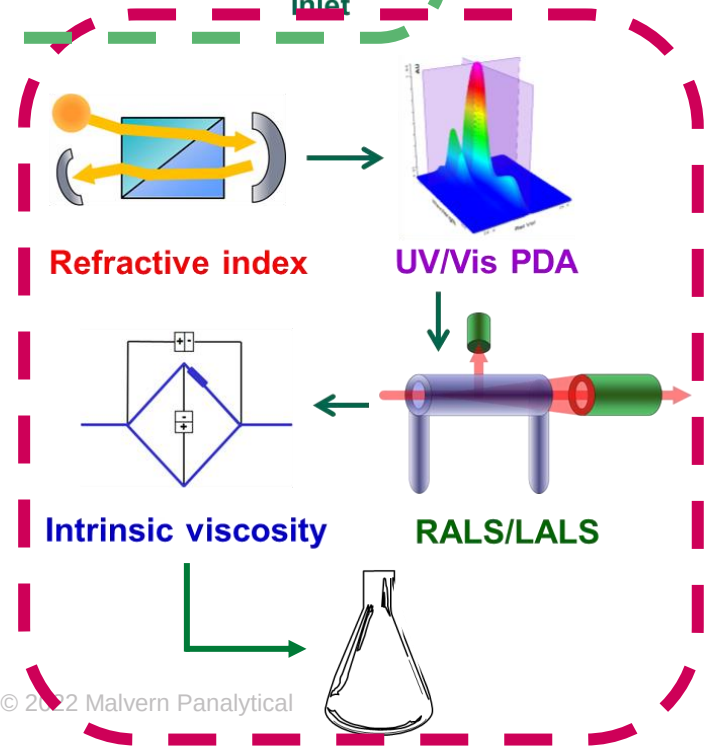

OMNISEC ULTRA

MULTI-DETECTOR SIZE EXCLUSION CHROMATOGRAPHY SYSTEM

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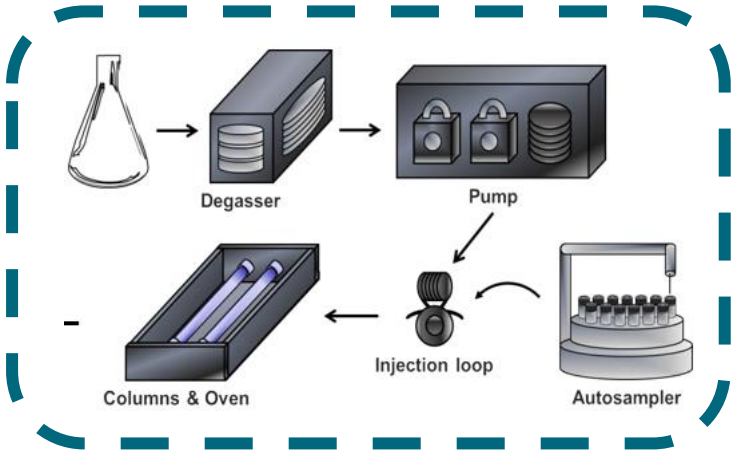
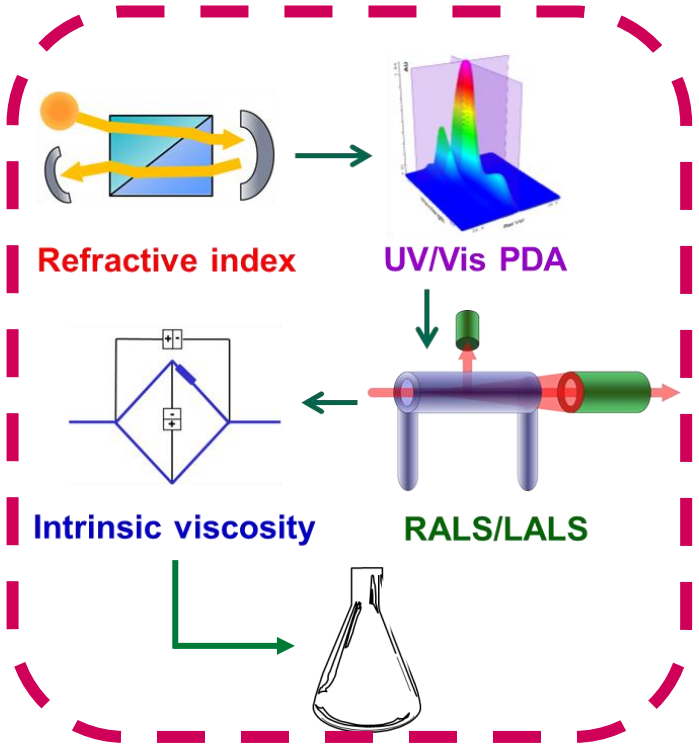
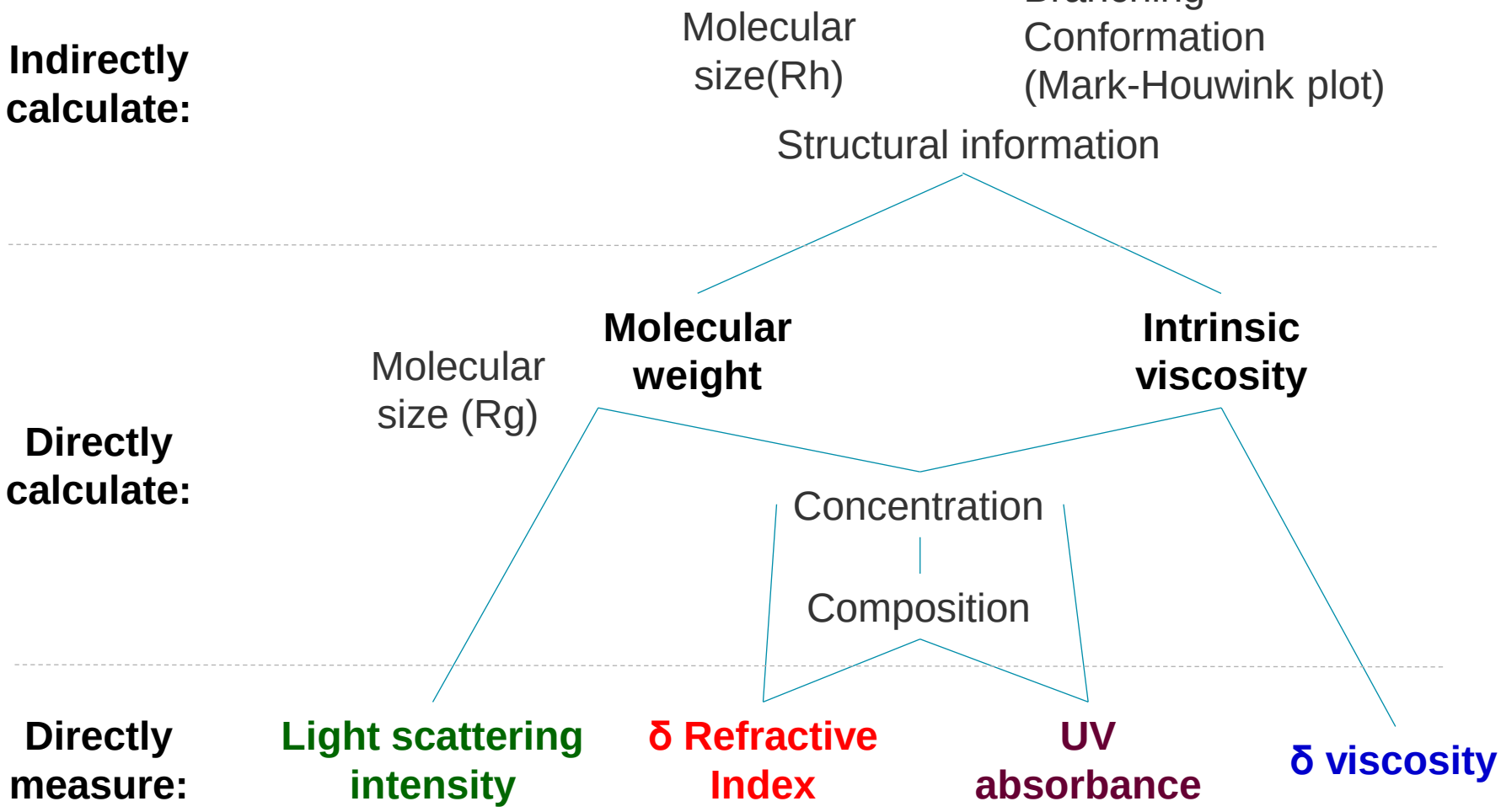


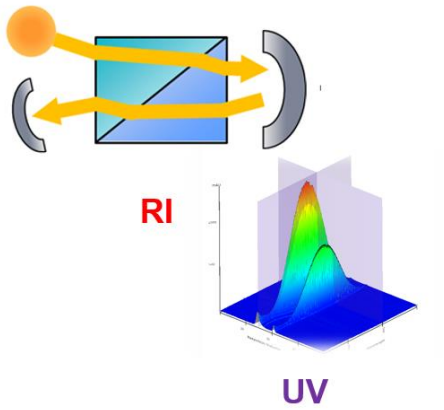


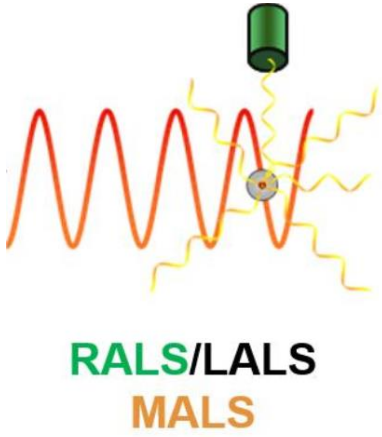
The multi-detection pyramid

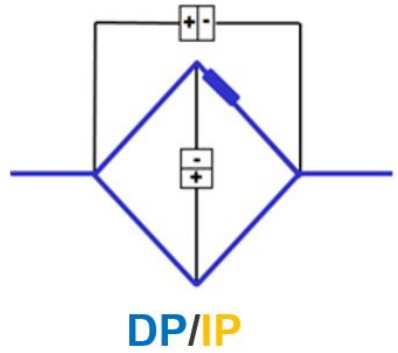


Detectors

OMNISEC Reveal







CONCENTRATION DETECTORS

- Differential Refractometer
- Diode-array-based UV/Vis Spectrometer
- RI and UV/Vis respond to sample concentration

LIGHT SCATTERING DETECTOR

- RALS 90° angle
- LALS 7° angle
- MALS 20 angles
- It responds to the sample molecular weight.

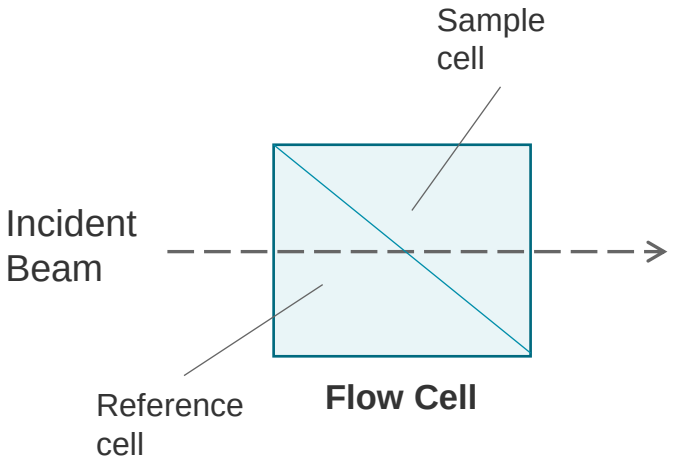
VISCOMETER DETECTOR

4-capillary Wheatstone bridge

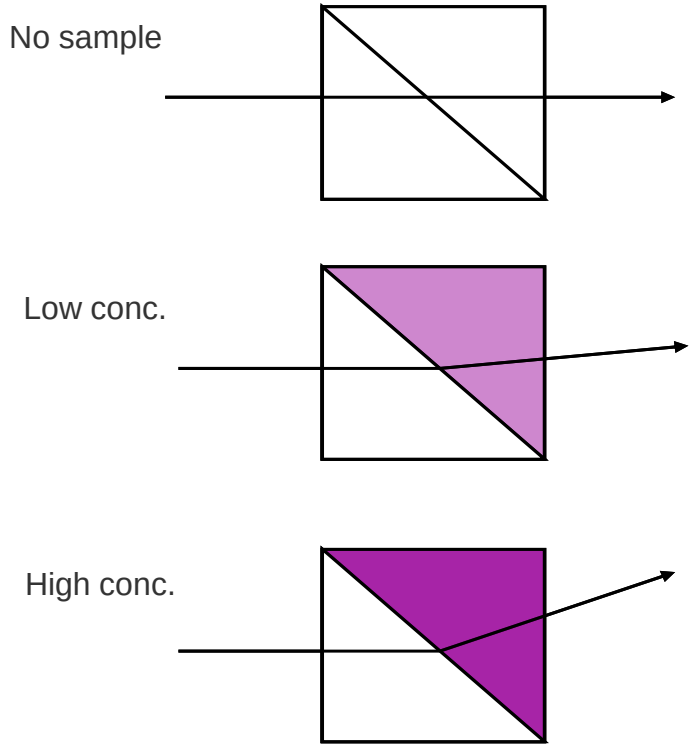
- It responds to the intrinsic viscosity of the sample in solution.

Differential Refractometer (RI)

Principle: Light travels at different speeds in different media.



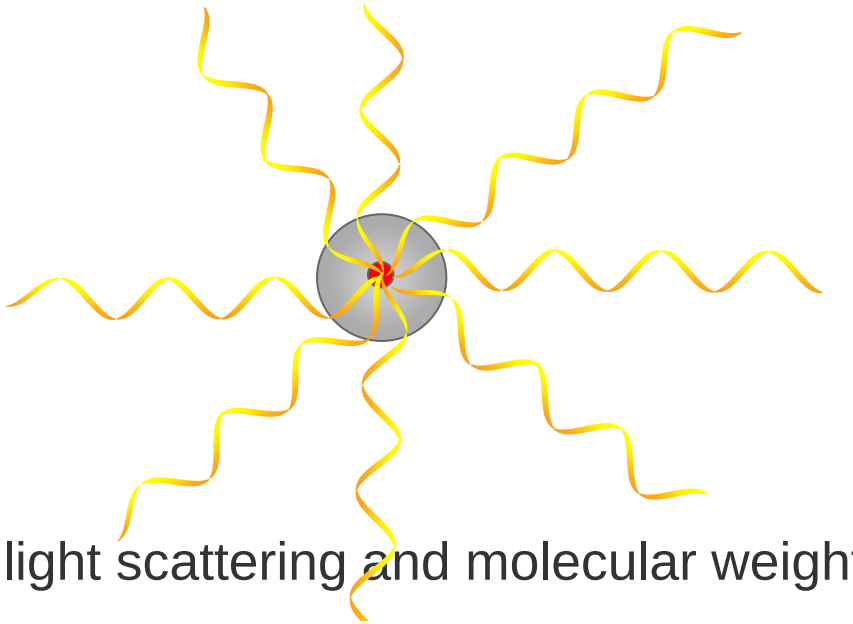
- Dissolving a solute in a solvent changes the magnitude of light refraction by the solution
- Deflection of beam corresponds to sample concentration



RI Output (mV) = K_{RI} • dn/dc • Concentration

Static Light Scattering

- A photon from an incident beam is absorbed by a macromolecule and re-emitted in all directions

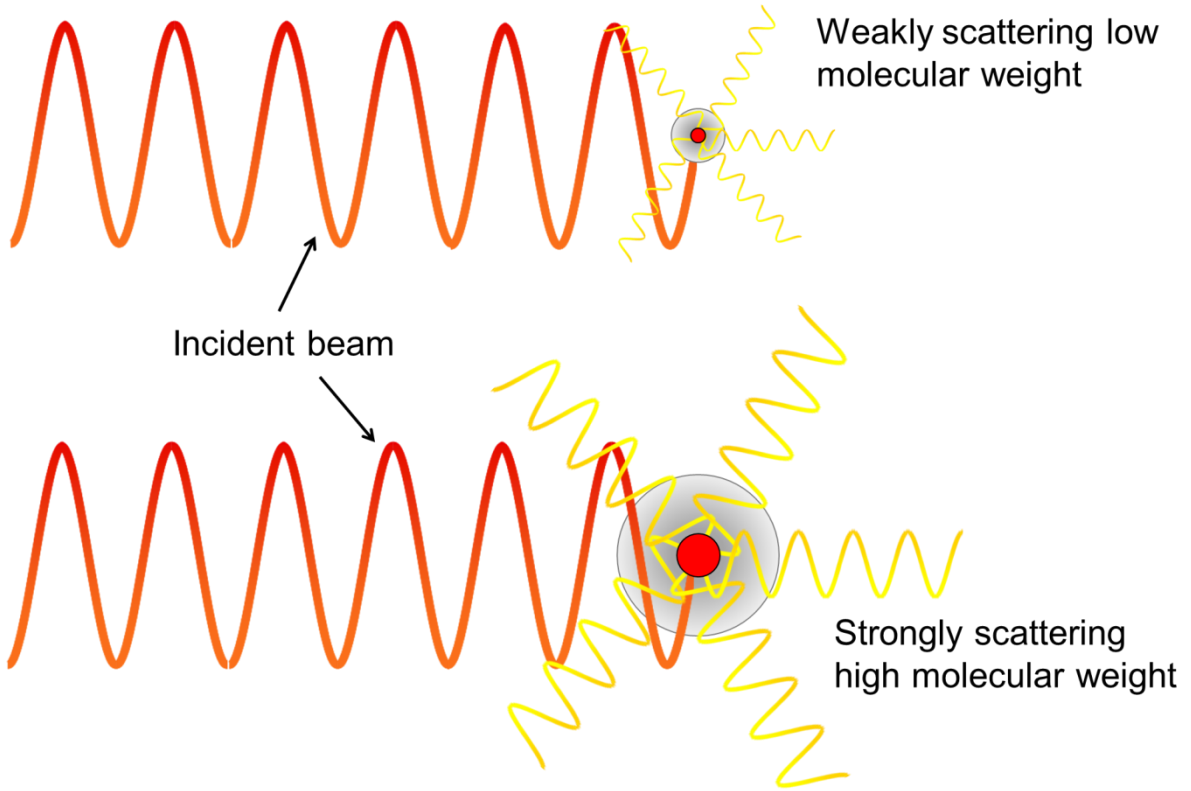


- The relationship between light scattering and molecular weight is defined by the Rayleigh equation:

$$R_\theta \propto \mathrm{Mw}$$

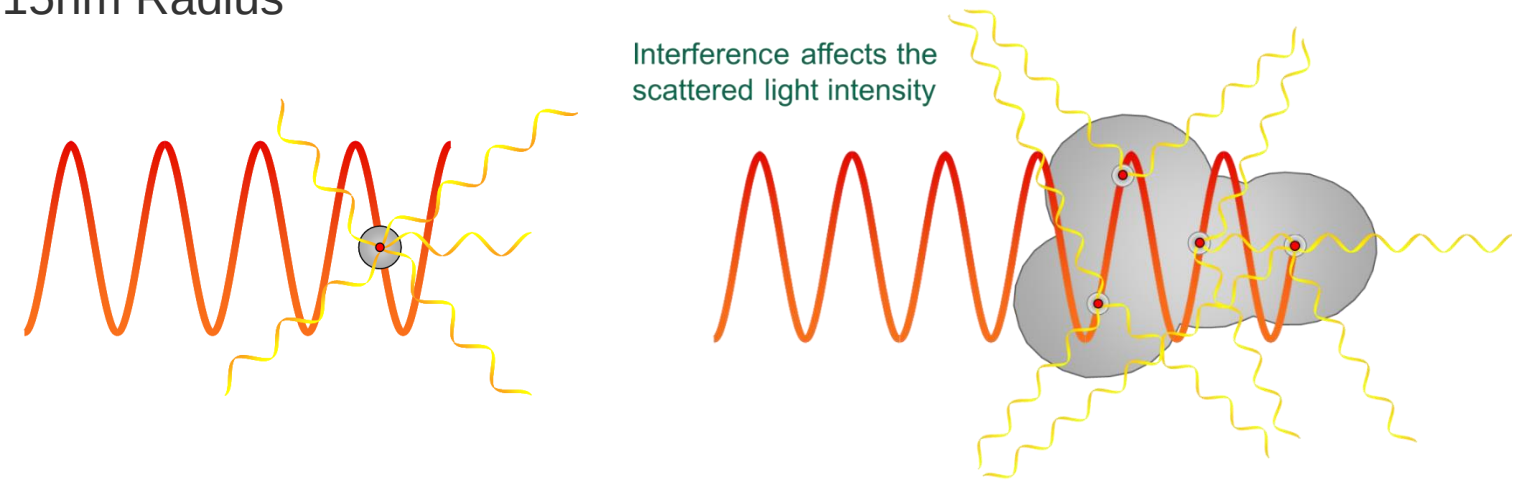
Light Scattering Theory

- The Rayleigh equation can be used to measure molecular weight by measuring the intensity of the light scattered by the sample if all the other parameters are known



Angular dissymmetry

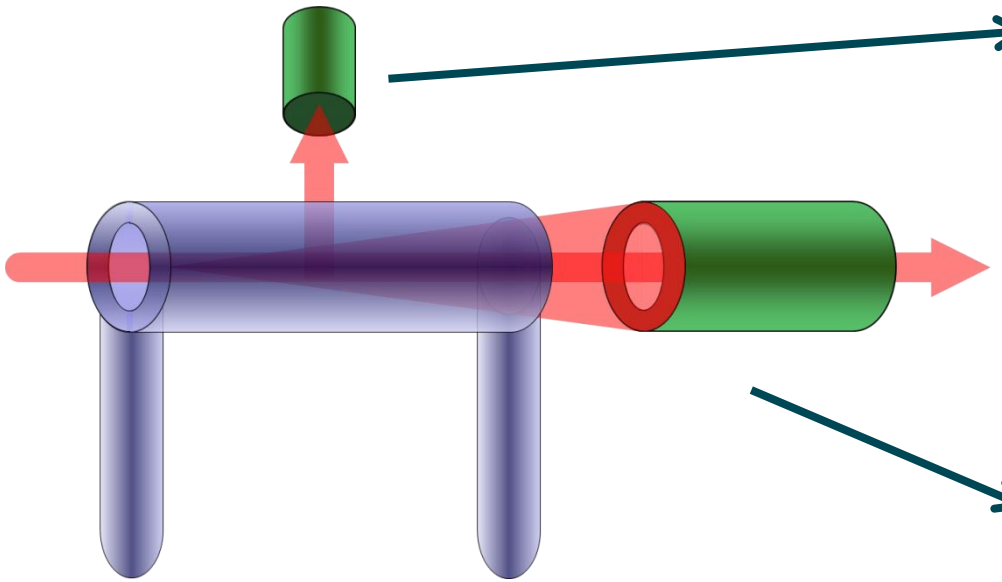
- Different molecules scatter light in different directions with different intensity
  - Smaller molecules scatter light evenly in all directions (isotropic scattering) <15nm Radius
  - Larger molecules scatter light in different directions with different intensities (anisotropic scattering) >15nm Radius



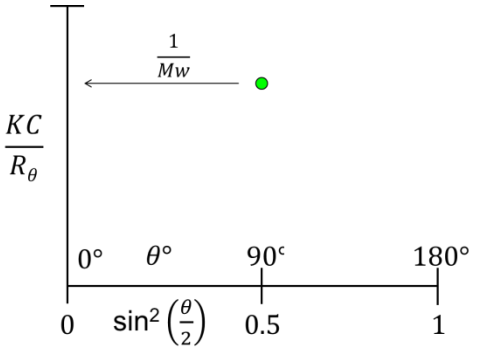
- We must account for the anisotropic scattering in some way in order to calculate the correct molecular weight
  - The Rayleigh equation tells us that if θ = 0 then the scattered light intensity relates directly to the sample's molecular weight
  - We can't measure at θ = 0 because the incident light is too bright

RALS/LALS

A RALS/LALS detector has the sensitivity of RALS for small molecules AND can account for anisotropy for large molecules

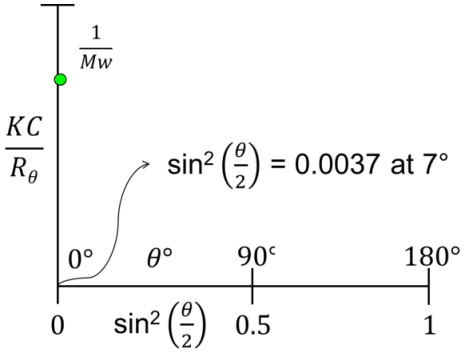


Small molecules

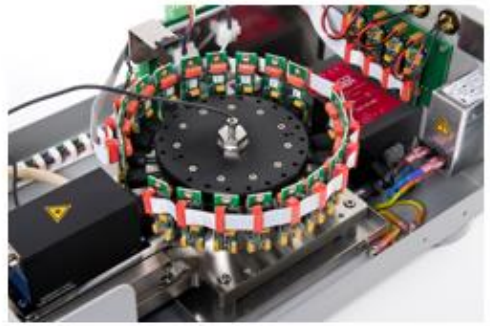


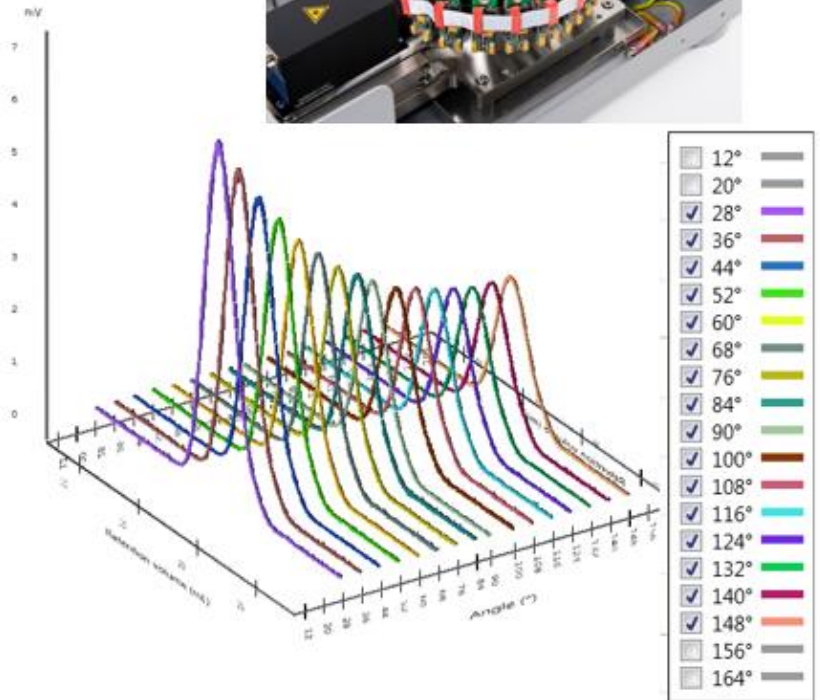
Right angle has the best signal to noise levels and thus the best sensitivity

Large molecules



Incredibly low angle means no need for data fitting or extrapolation with minimised errors





Multi-Angle Light Scattering

SEC-MALS 20 angles

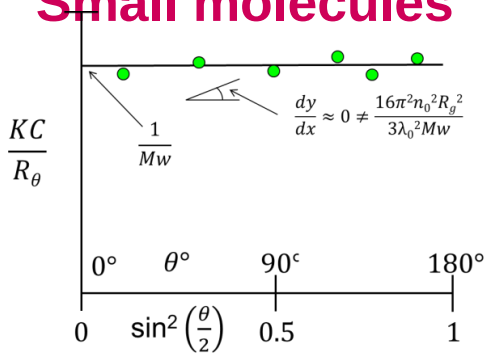
- A modular multi-angle light scattering system with 20 measurement angles
- Works with other Viscotek system and OMNISEC
- Interfaces with 3rd party SEC systems
- The SEC-MALS includes 20 detector angles

Multi-Angle Light Scattering

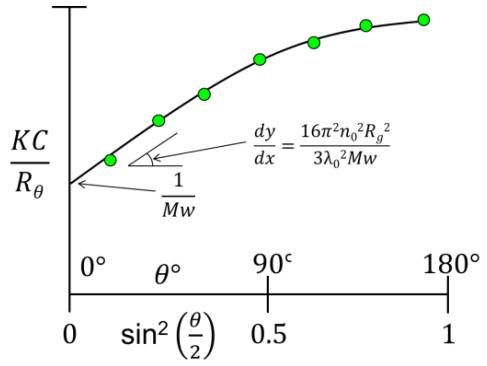
SEC-MALS 20 angles

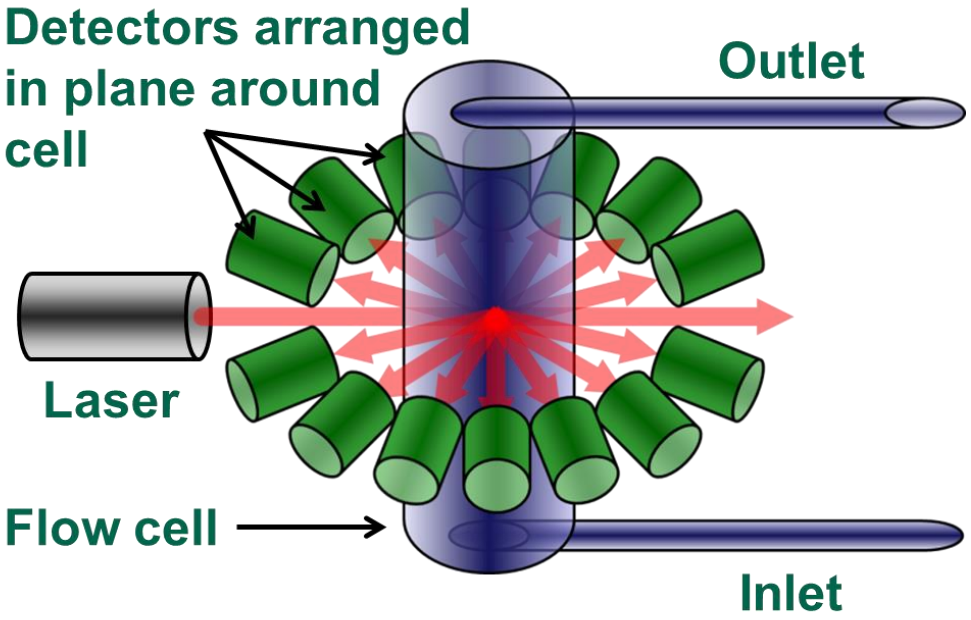
- MALS measures the scattered light intensity at many angles then extrapolates back to 0°
- Anisotropy of the scattered light is accounted for by the extrapolation but is dependent on the fit
- MALS works for all molecules
- R_g can only be measured for larger molecules

Small molecules



Large molecules

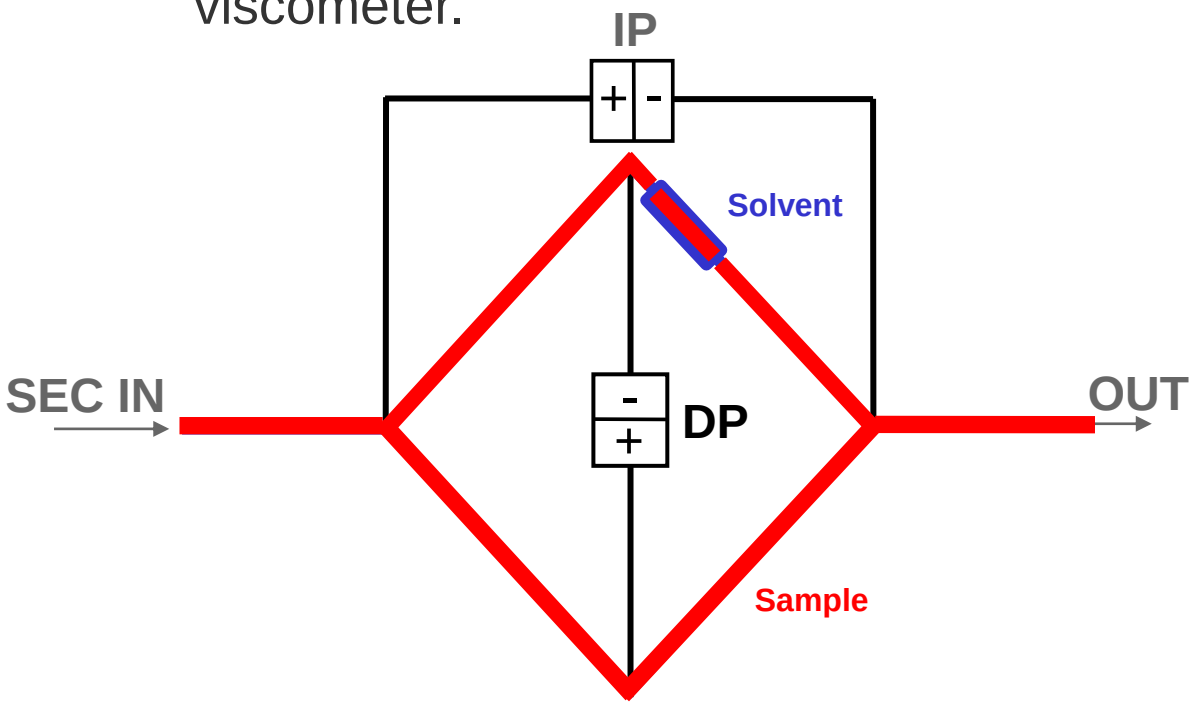


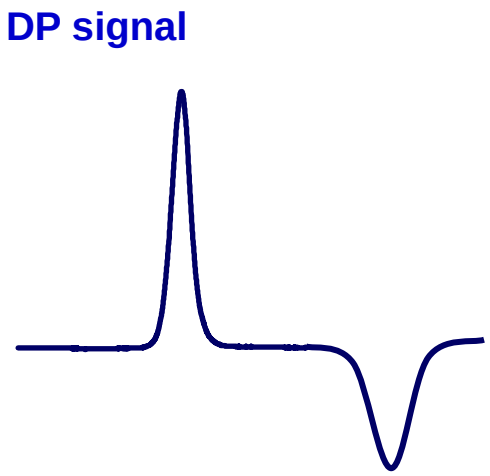


How do we measure IV?

4-capiliary Viscometer Bridge - The Wheatstone Bridge Concept

- The viscometer detects changes in pressure when the sample travels though the viscometer.





$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0}$$

$$\eta_{sp} = \frac{4\,DP}{IP - 2DP} = C \cdot IV$$

Relationship of the output from the pressure transducers and specific viscosity

Relationship of the specific viscosity and intrinsic viscosity

How can we relate IV to structure?

Intrinsic viscosity has the units:

$$dL/g$$

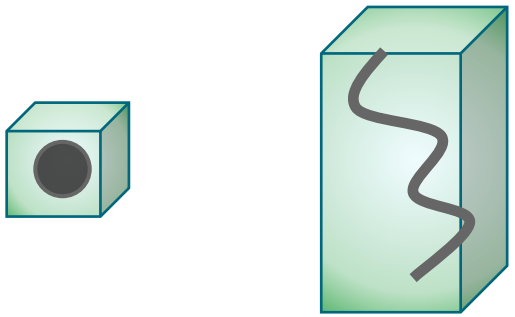
Intrinsic viscosity is inversely proportional to molecular density:

$$IV \propto \frac{1}{density}$$

We can look at structure in these terms:

$$IV \propto \frac{volume}{mass}$$

Which of these two molecules with the same mass occupies the largest volume of space?



Size measurements - R_h

Hydrodynamic Radius (R_h)

Triple Detection SEC/GPC – IV and M_w

R_h is the radius of an equivalent solid sphere that increases the fluid viscosity by the same amount as the macromolecule.

$$[\eta]M = \frac{10}{3}\pi \cdot N_A \cdot R_h^3$$

IV M_w Constants

Triple Detection

- Analyze hydrodynamic size from < 1 nm to the exclusion limit of the SEC column (~200 nm)
- No extrapolation or fitting parameters

Dynamic Light Scattering (DLS) – Zetasizer products

R_h is the radius of an equivalent sphere that diffuses with the same speed as the molecule of interest.

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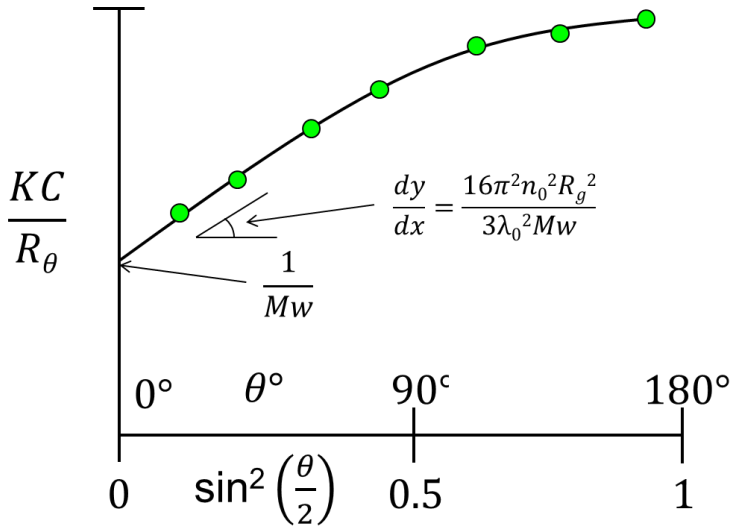
Size measurements - R_g

Radius of Gyration (R_g)

Rg is the root-mean-square of the radii from the centre of the mass to the different mass cores within the molecule.



- Direct measurement by changes in scattered light intensities with observation angle
  - RALS/LALS
  - MALS

Limitations:

- Requires good S/N light scattering signal
- Lower size detection limit = 10-15 nm
  - Limit of Anisotropic scattering
- Large structures require non-linear curve fitting

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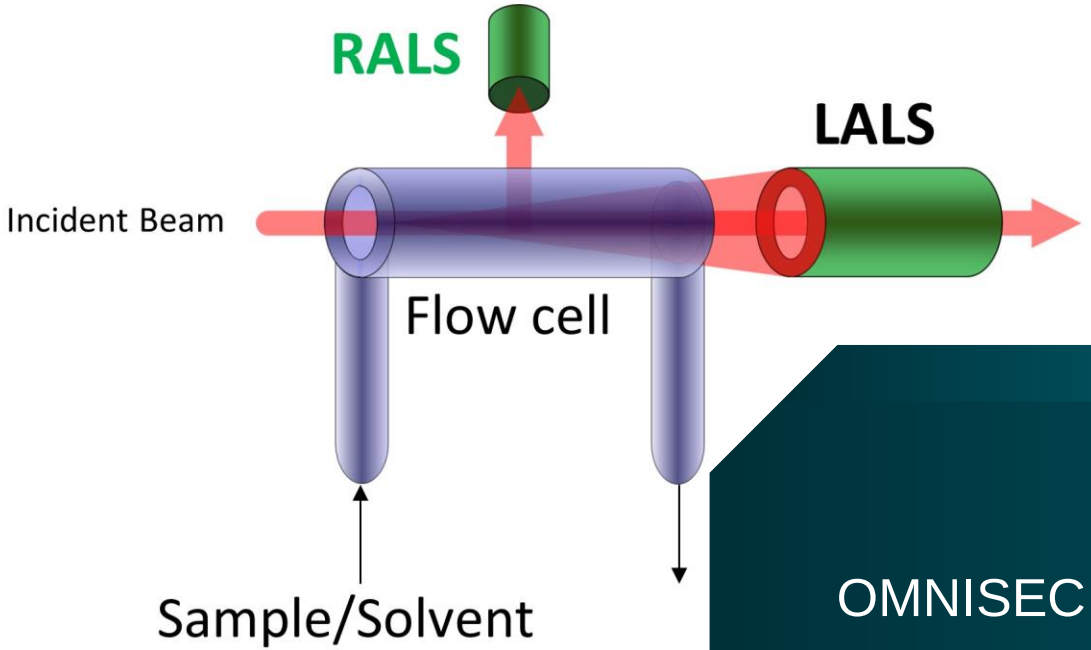
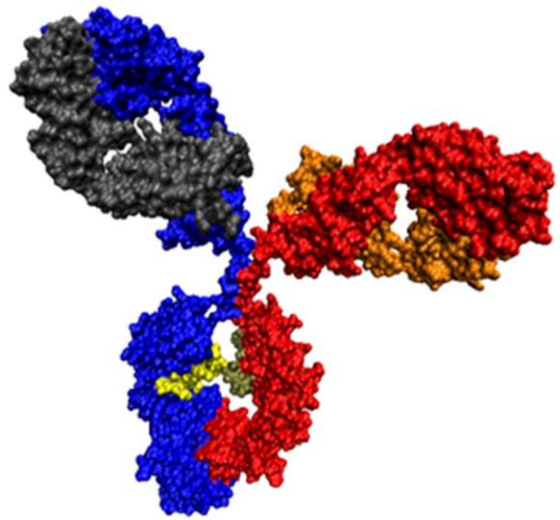
RALS: **R**ight **A**ngle **L**ight **S**cattering

LALS: **L**ow **A**ngle **L**ight **S**cattering





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RALS/LALS

Antibody applications

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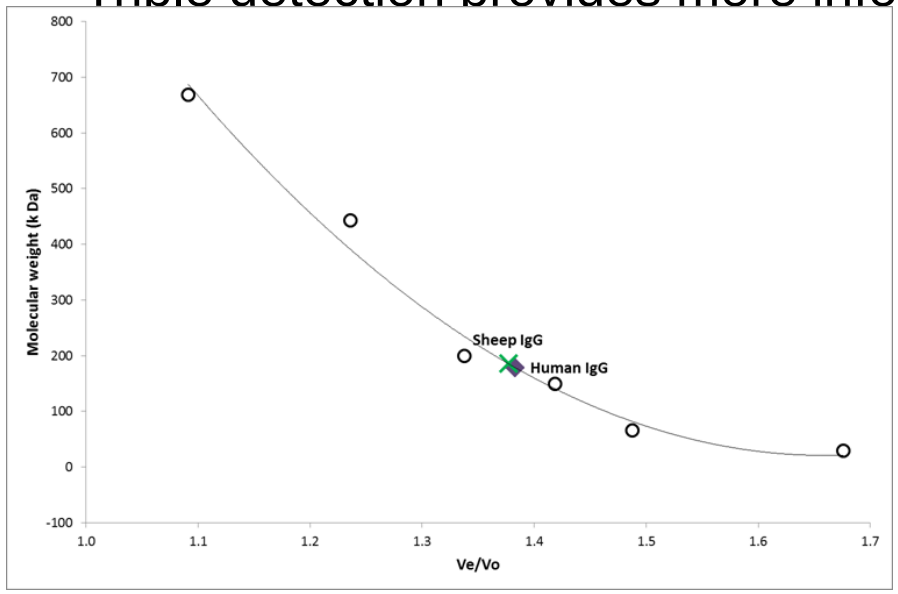
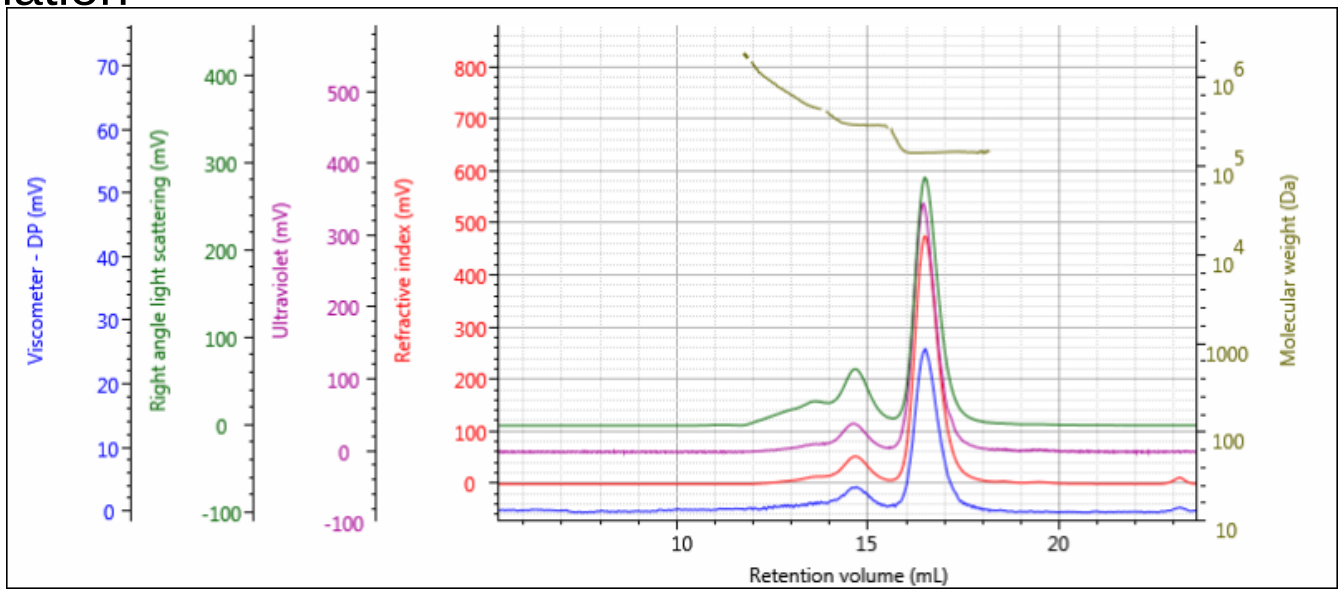
Conventional vs Multi-detection

- MW overestimated in conventional calibration
- Antibodies do not have a globular structure, thus the retention times compared to globular standards introduce larger disparities between the estimated and ‘true’ MW
- Triple detection provides more information

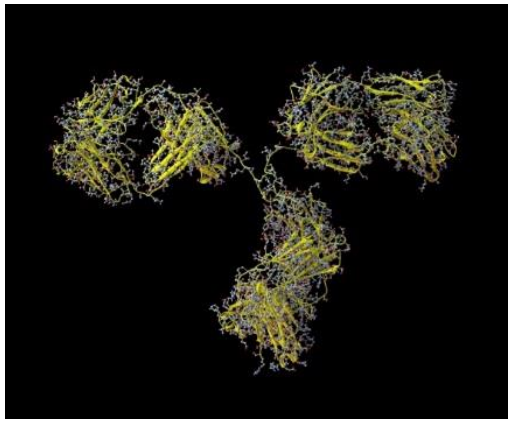


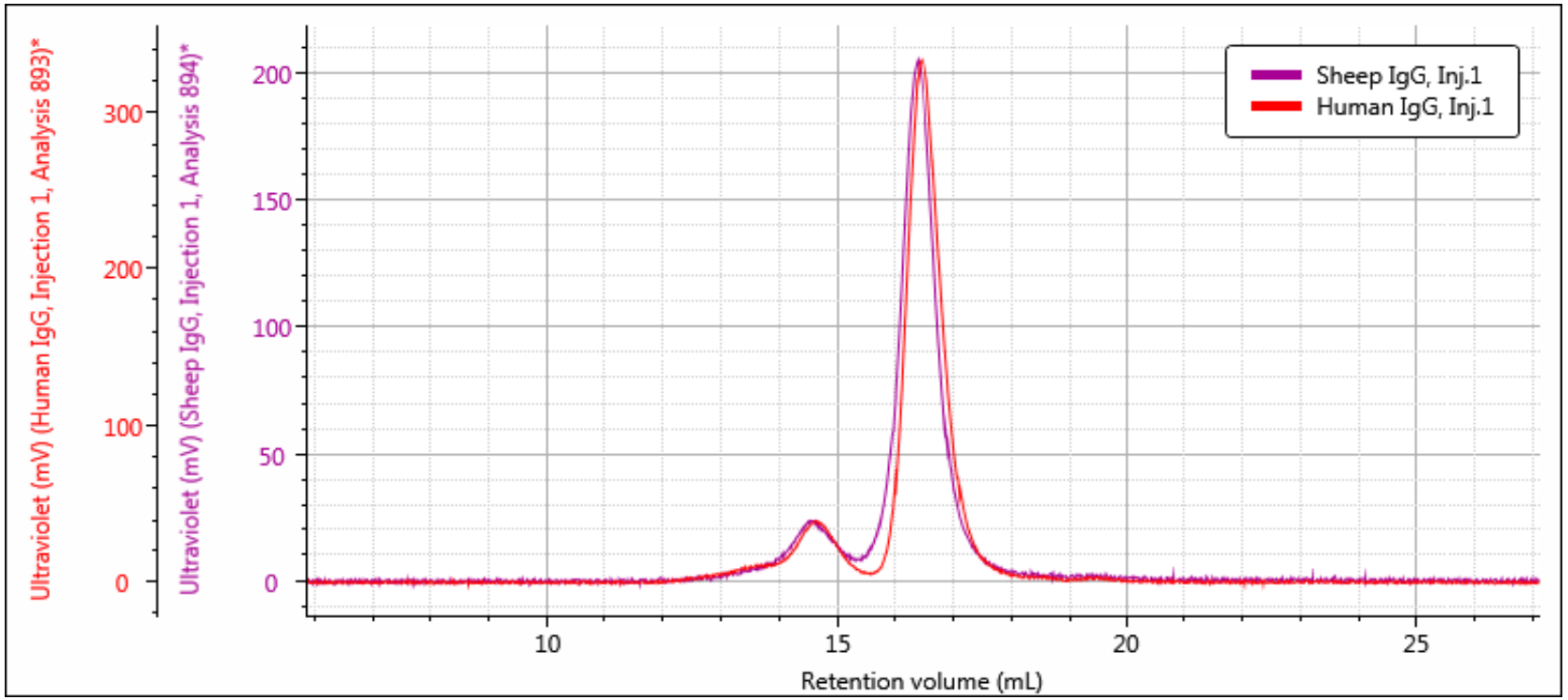


Sheep & Human IgG

Overlay if RI responses

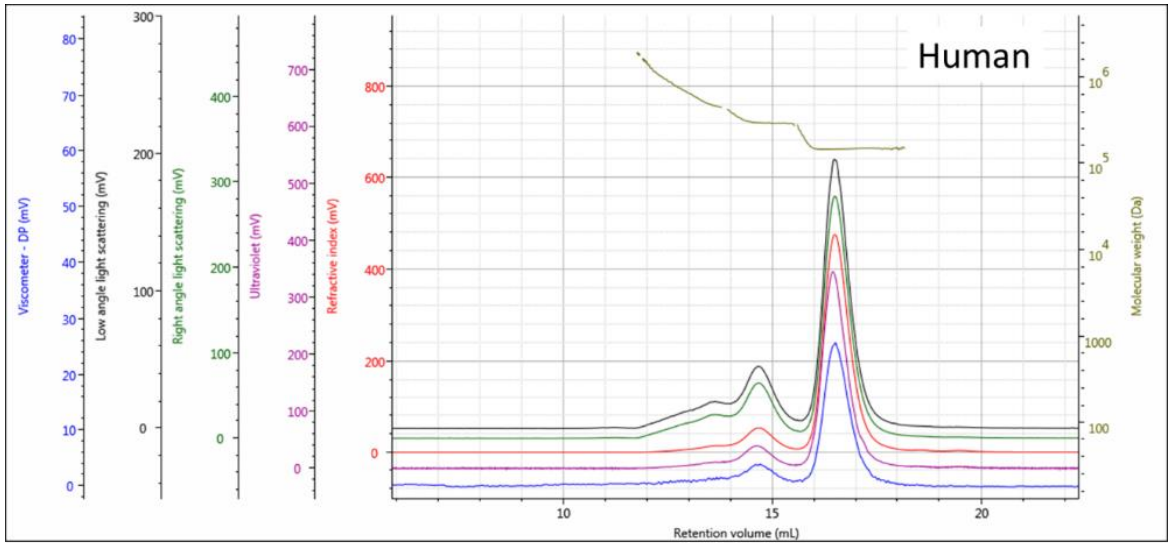
- Small compositional differences between the two samples are clear





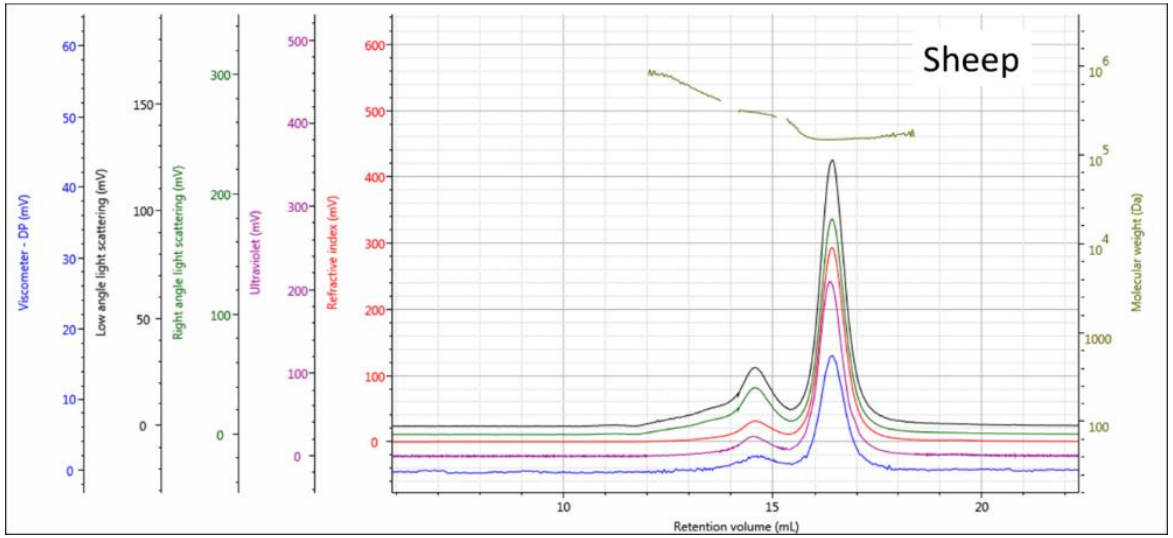
Sheep & Human IgG

Human IgG



	monomer	dimer	trimer	other
Mw (kDa)	147.2	307.3	481.2	791.2
% composition	70	19	6	5
Pd	1.0012			
IV	0.0569			
Rh	5.1			

Sheep IgG:



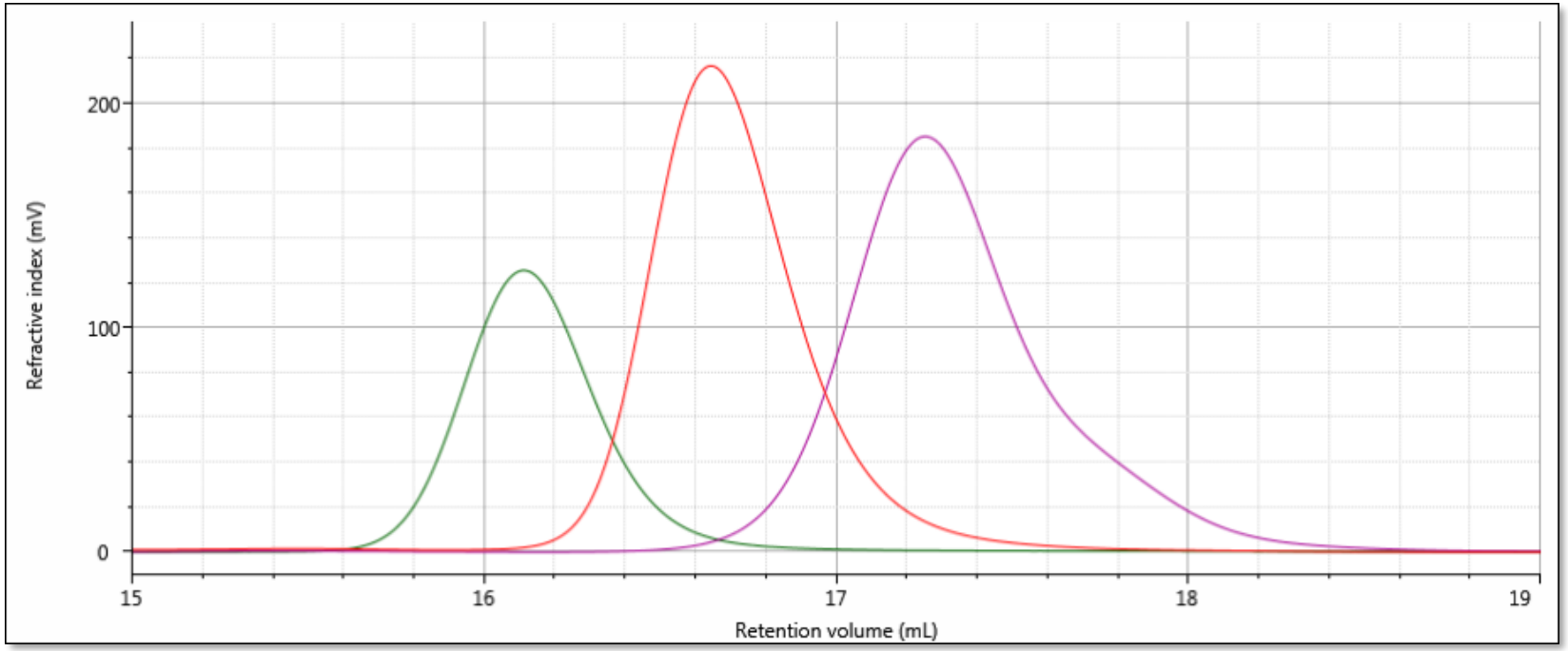
	monomer	dimer	other
Mw (kDa)	153.2	303.8	552.1
% composition	86	11	3
Pd	1.0026		
IV	0.0595		
Rh	5.25		

Multi-detection: IgG

- Mw of ~150 kDa is more in line with expectations.
- The monomer peak of Human IgG is less polydisperse – better resolution between monomer and dimer peaks.
- There are compositional differences that can be quantified. Human IgG is more aggregated i.e. lower yield monomer.
- The monomer Rh of sheep IgG is larger than that of Human IgG which rationalises the differences in observed retention times.

Analysis of three antibodies

- Three antibodies eluting at different times in the chromatogram
- They have different molecular sizes but do they have different molecular weights?



Column calibration vs. advanced detection

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Column calibration

Sample ID	Mw (Da)	Mw/Mn
1	143,209	1.021
2	96,863	1.035
3	201,996	1.029

Advanced detection

Sample ID	Mw (Da)	Mw/Mn	IV (dL/g)	Rh (nm)
1	149,300	1.000	0.065	5.37
2	151,100	1.000	0.062	5.29
3	150,000	1.002	0.070	5.49

- Column calibration data ties molecular weight to retention volume → samples that elute earlier have higher molecular weight
- Advanced detection uses light scattering to measure molecular weight independently of retention volume → absolute molecular weight
- Even though the three antibody samples have different molecular sizes, their molecular weight of all three samples is 150 kDa

Biosimilars – stress testing

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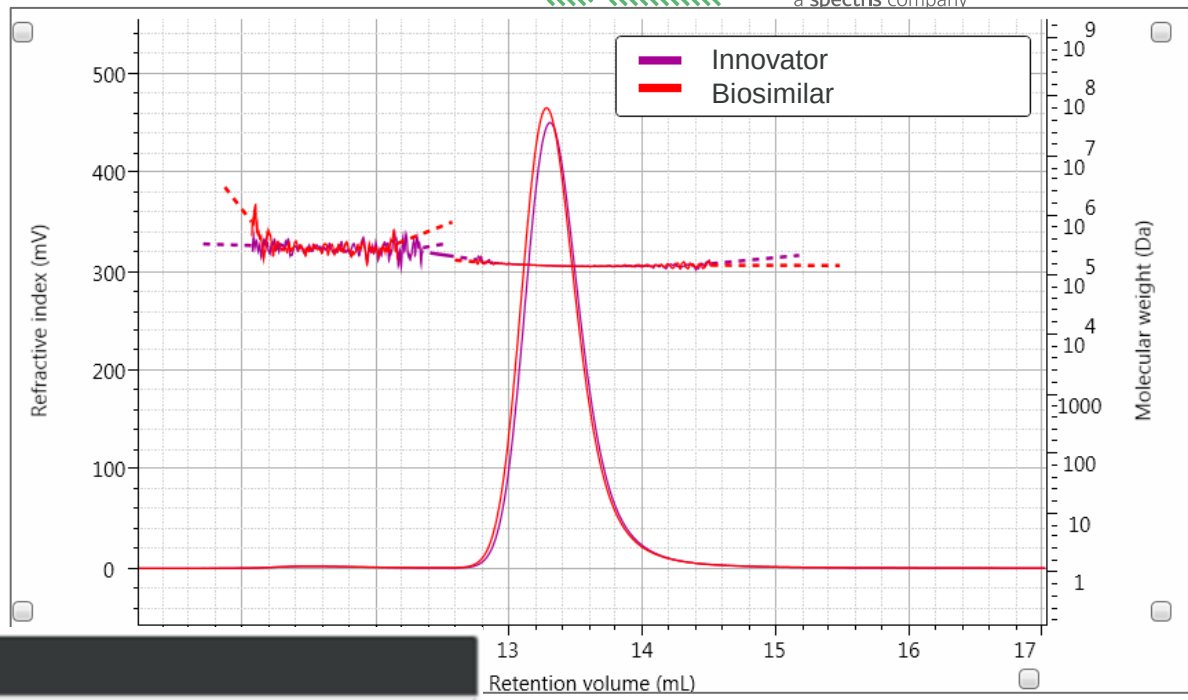
Biosimilars

- Biologicals are used for treatment of a wide range of diseases.
- Some of the most popular biologicals are coming off patent opening up the opportunity for the development of biosimilars
- Selection of approved
  - Bevacizumab (Avastin®)
  - Denosumab (Prolia® and Xgeva®)
- Biosimilars have been shown to be biologically similar to innovator products
  - Produce the same clinical response
  - Shortened licensing pathway

- To prove biosimilarity FDA requires the use of state-of-the-art analytical instruments
- Multi-detector SEC is a key tool in proving biosimilarity
  - Absolute Mw
  - Dispersity (Mw/Mn)
  - Oligomeric state
  - Formulation effects, purity, stability, product and process related stress

Denosumab

- Innovators commercially sold as Prolia® and Xgeva® with Mw of 147kDa
  - Reduce the risk of broken bones in people with osteoporosis
- Under the same conditions the innovator and biosimilar show minimal differences in Mw, Dispersity and Rh.

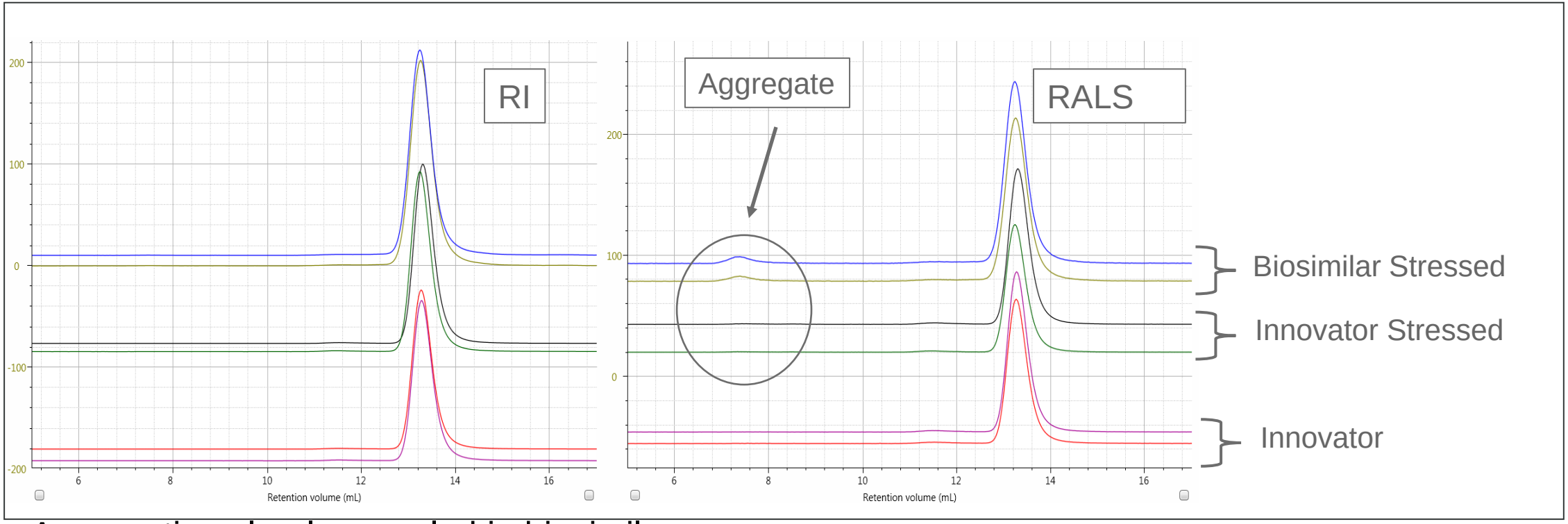


Results by sample and peak.

Parameter	Denosumab Biosimilar		Denosumab Innovator	
	Peak 1	Peak 2	Peak 1	Peak 2
RV (mL)	11.48	13.29	11.37	13.32
Mw (g/mol)	363,000	146,000	290,500	146,500
Mw/Mn	1.174	1.001	1.031	1.001
Frac. of sample (%)	0.756	99.24	0.8089	99.19
Rh(η)w (nm)	N/C	4.211	N/C	4.294

Denosumab Stress testing

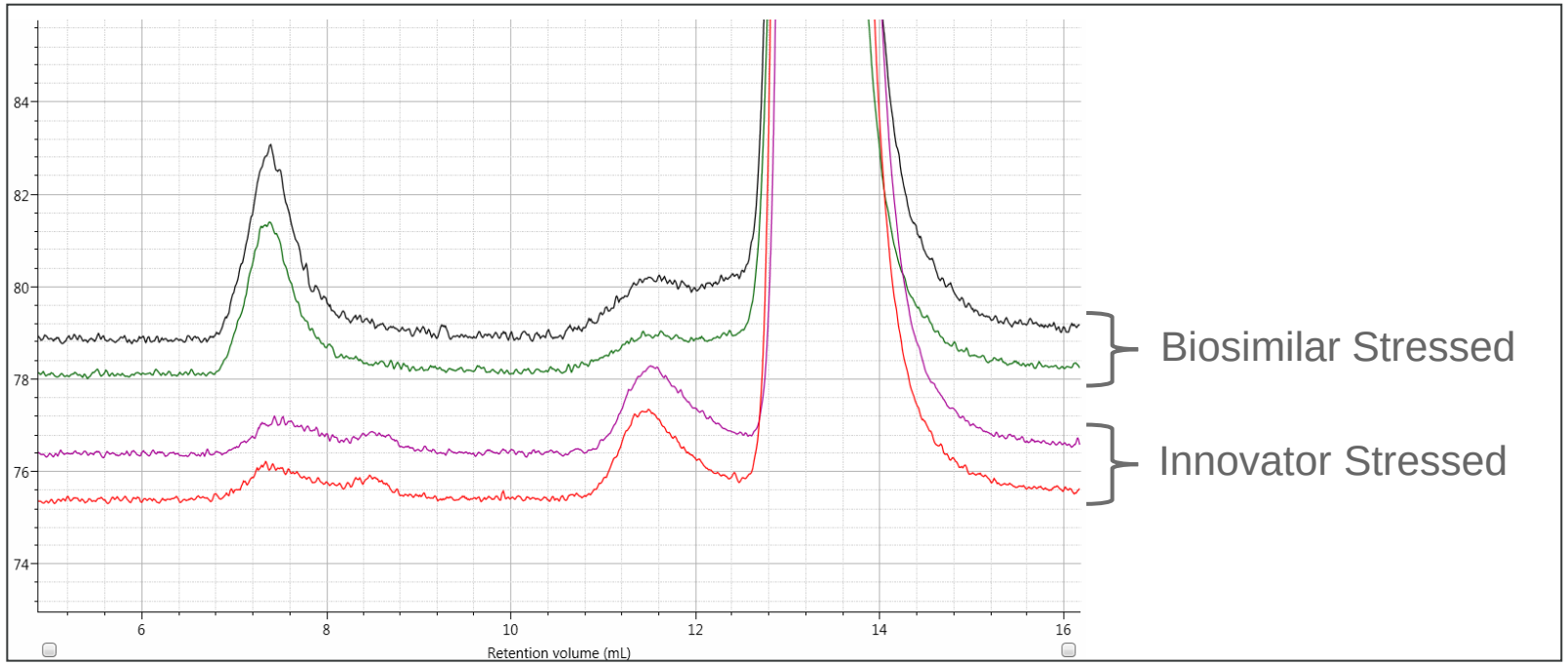
- Denosumab innovator and biosimilar were incubated at temperature for an extended period
- RI and RALS overlay of denosumab innovator, innovator stressed and biosimilar stressed.



- Aggregation clearly revealed in biosimilar

Denosumab Stress testing

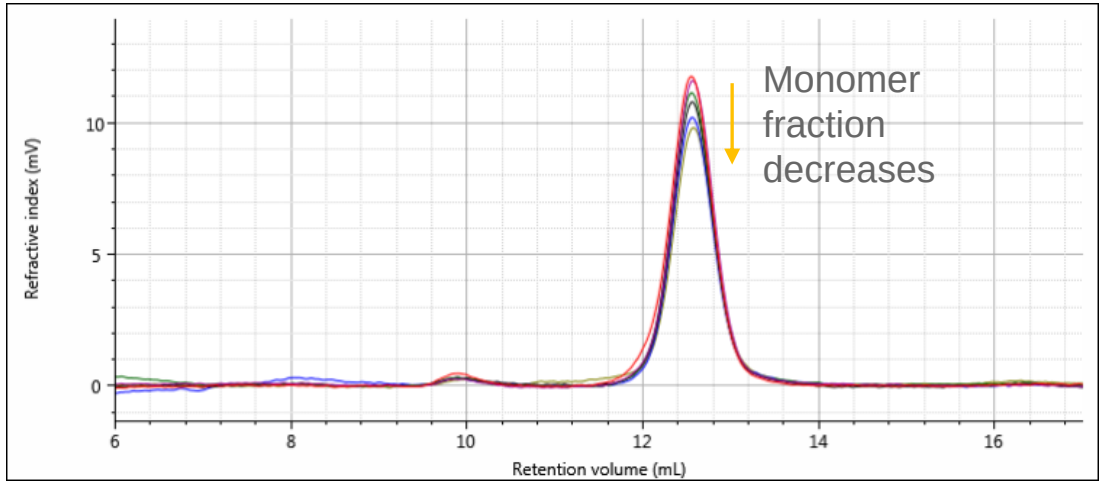
- Magnification of the RALS signal highlights the difference between the innovator and biosimilar
  - More HMW aggregation in biosimilar
  - More dimer in the innovator
  - Differences in aggregate structure – could indicate a different route to aggregate formation.

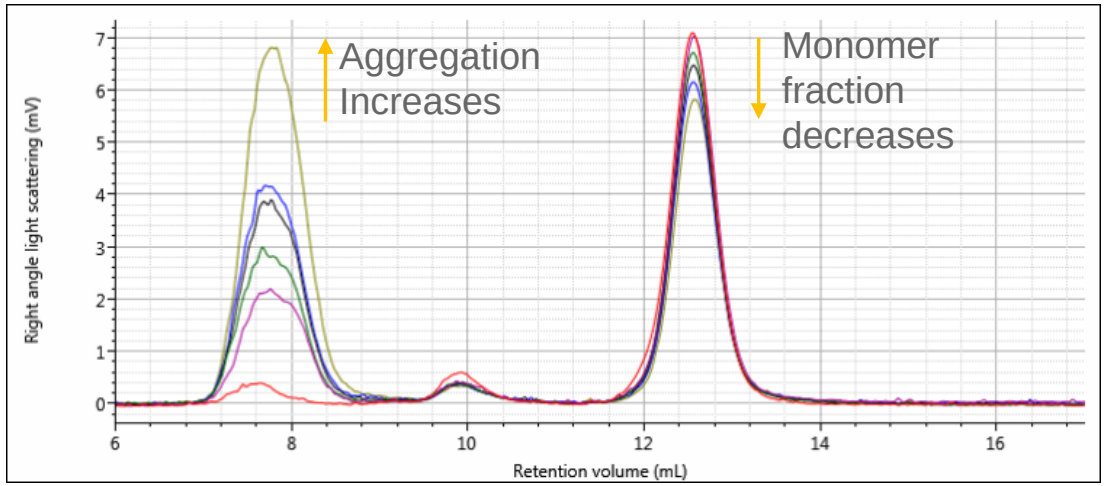


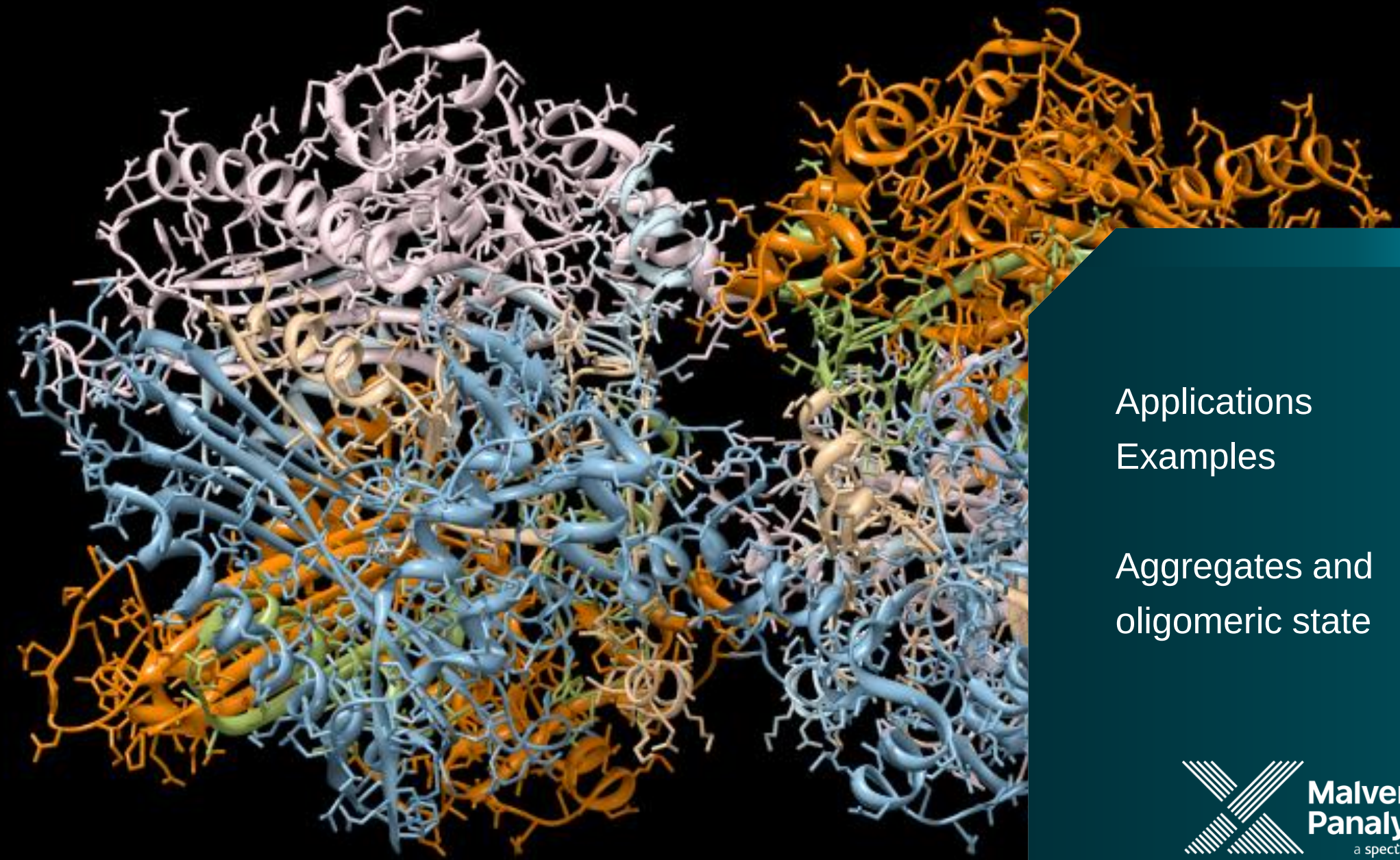
Bevacizumab

Thermal stress

- Samples incubated at 60°C for 4 hours
  - Malvern MicroCal DSC suggests the onset temperature for denaturation lies between 60-63°C
- Monomer fraction decreases in the RI/UV signal
- RALS detector clearly identifies significant increase in aggregation of Bevacizumab which were not identified using a single concentration detector only
- Calibrated system shows monomer concentration decreases from 0.162mg/mL → 0.138mg/mL







Applications
Examples

Aggregates and
oligomeric state

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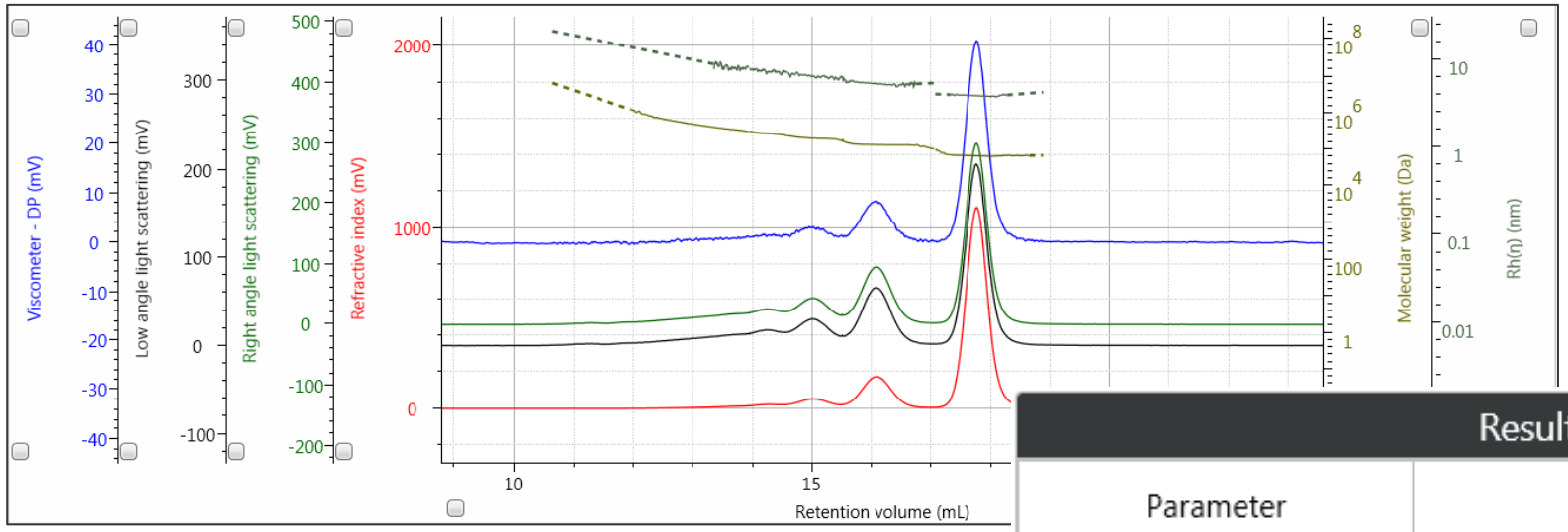
BSA molecular weight using OMNISEC

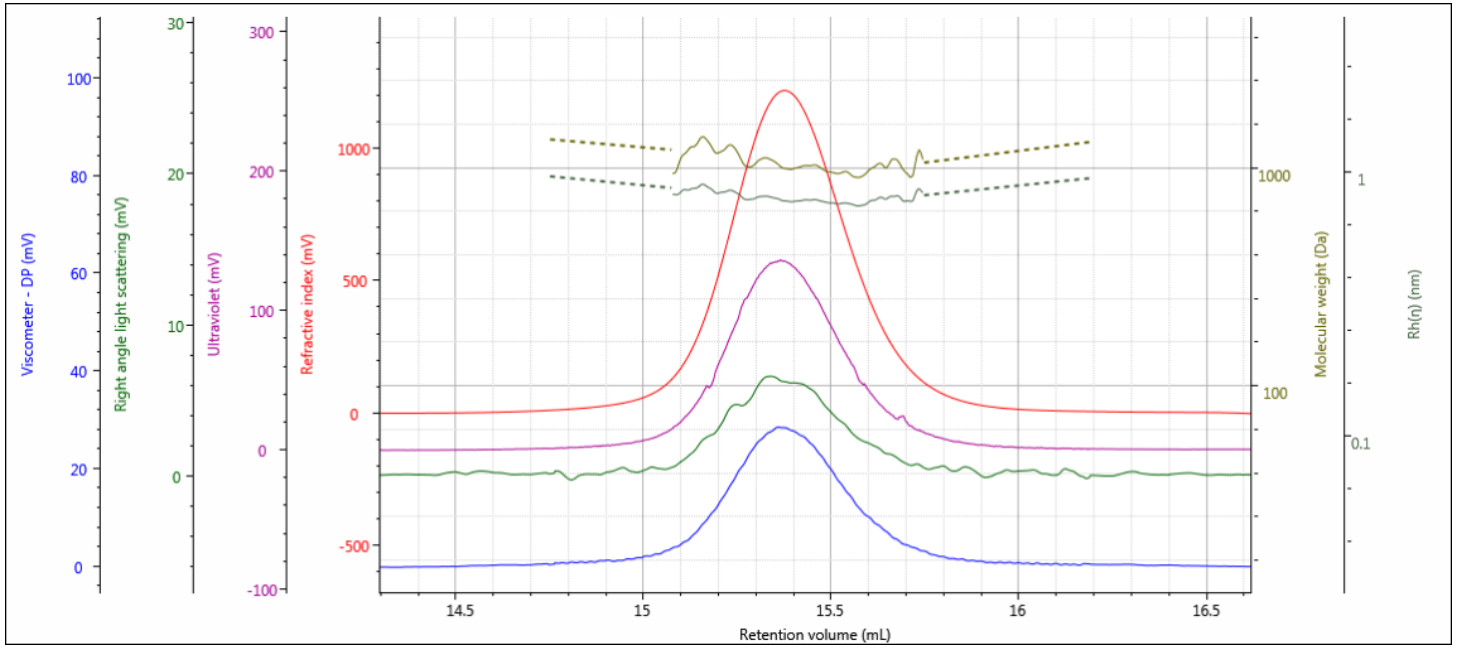
- Bovine serum albumin is the standard protein used to check system performance
- This sample contains monomer, dimer, trimer, tetramer and aggregates



Results by sample and peak.					
Parameter	Inj. 3 BSA Standard 21/03/2017 17:08:02				
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
RV (mL)	13.71	14.3	15.03	16.1	17.77
Mw (g/mol)	472,800	268,100	202,100	133,600	66,460
Mw/Mn	1.154	1.004	1.003	1.002	1.001
Frac. of sample (%)	1.946	2.038	5.236	16.37	74.41
Rh(η)w (nm)	9.138	7.241	6.356	5.219	3.803

Peptide analysis - Bradykinin

- Bradykinin is used to lower blood pressure by vasodilation
- This peptide is known to contain 9 amino acids (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) with an Mw of 1061 Da.
- For peptides it is typically not possible to define a dn/dc, so one must be calculated using the RI detector.



Results by sample and peak.	
Parameter	Inj. 1 Bradykinin 04/09/2...
	Peak 1
RV (mL)	15.38
Mw (g/mol)	1,058
Mw/Mn	1.009
IVw (dL/g)	0.0293
Rh(η)w (nm)	0.7876
Rgw (nm)	N/C

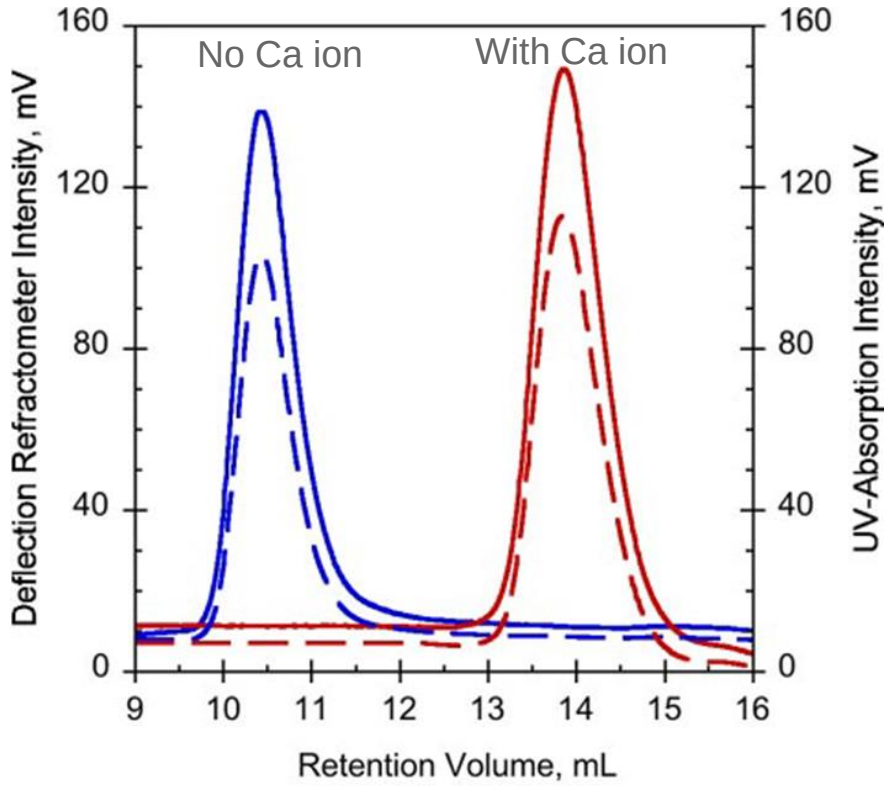
External Binding Factors

CONFORMATIONAL CHANGES, OLIGOMERIC STATE AND STOICHIOMETRY

Conformational Changes

Conventional SEC

- Adenylate Cyclase Toxin is an intrinsically disordered protein.
  - Major virulence factors of *Bordetella pertussis*, the causative agent of whooping cough
- Adopts ‘active’ form in the presence of calcium ions.
  - Polypeptide cofactor binding domain of cobra toxin, in the absence and presence of the calcium cofactor.
- Significant change in retention volume suggests
  - With Ca = Monomer
  - Without Ca = multimer



UV = solid RI = dash

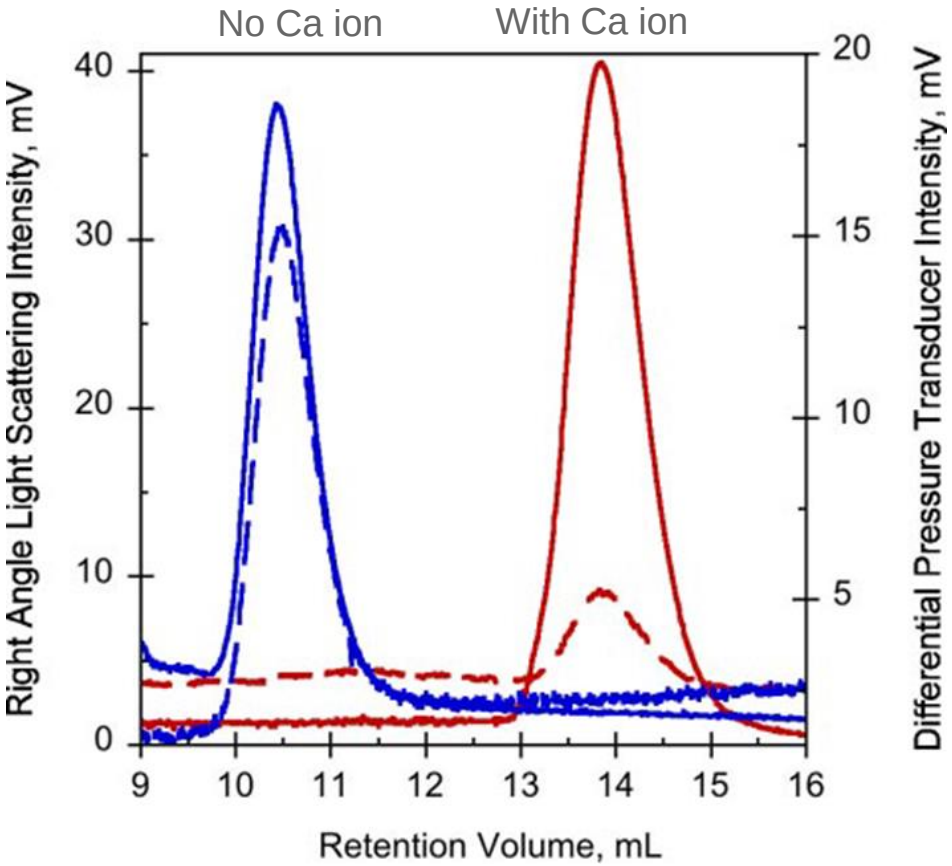
No Ca ion	With Ca ion
~600kDa	~100kDa

Conformational Changes

Multi-detection SEC

- Mw the in the presence or absence of Ca
- Significant differences in IV and size reflect significant structural changes upon calcium binding
  - Not multimer but different structure
- 7-fold decrease in IV, ~2-fold decrease in Rh

	No Ca ion	With Ca ion
Mw (kDa)	73.6	73.2
IV (dL/g)	0.35	0.055
Rh (nm)	7.4	4.0



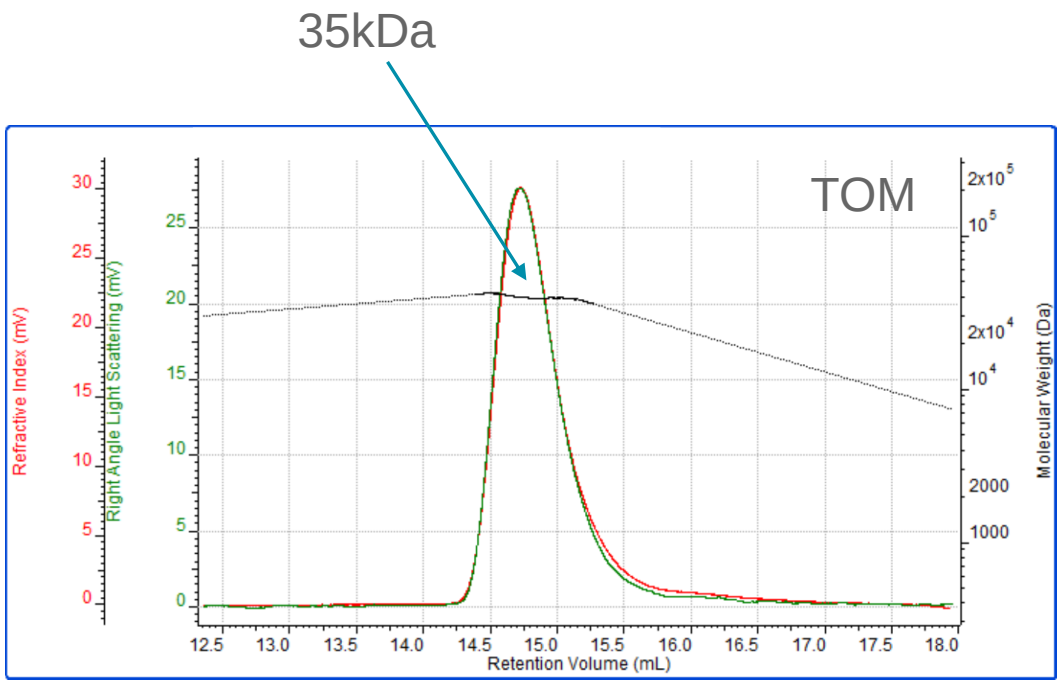
RALS = solid DP = dash

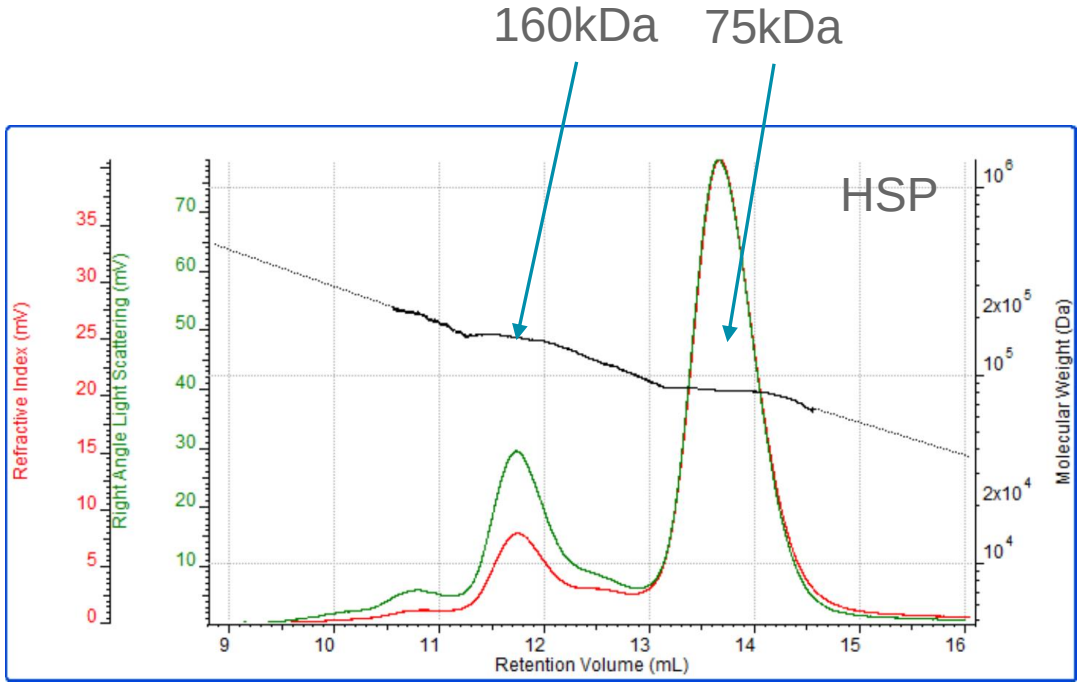
Paper: A. Chenal, J. Biol. Chem., 2009; 284: 1781 - 1789.

Oligomeric state and Stoichiometry

HSP and TOM

- TOM is a cochaperone of HSP in mitochondrial protein import
- Interaction between HSP and TOM is ATP dependent

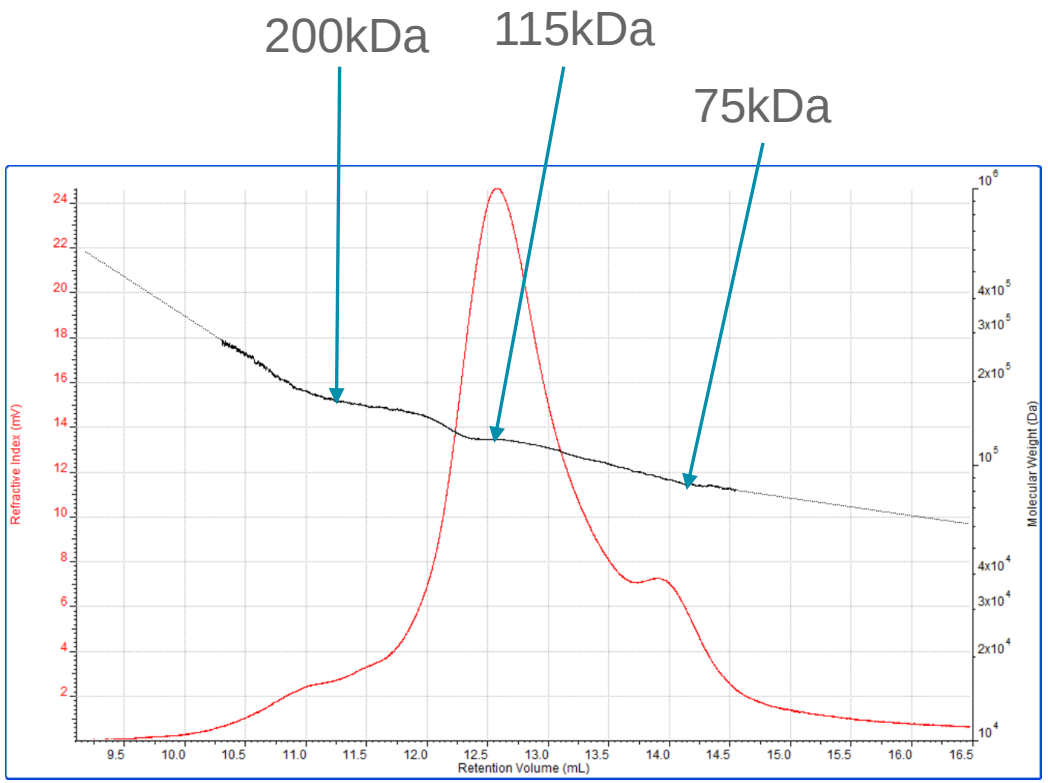


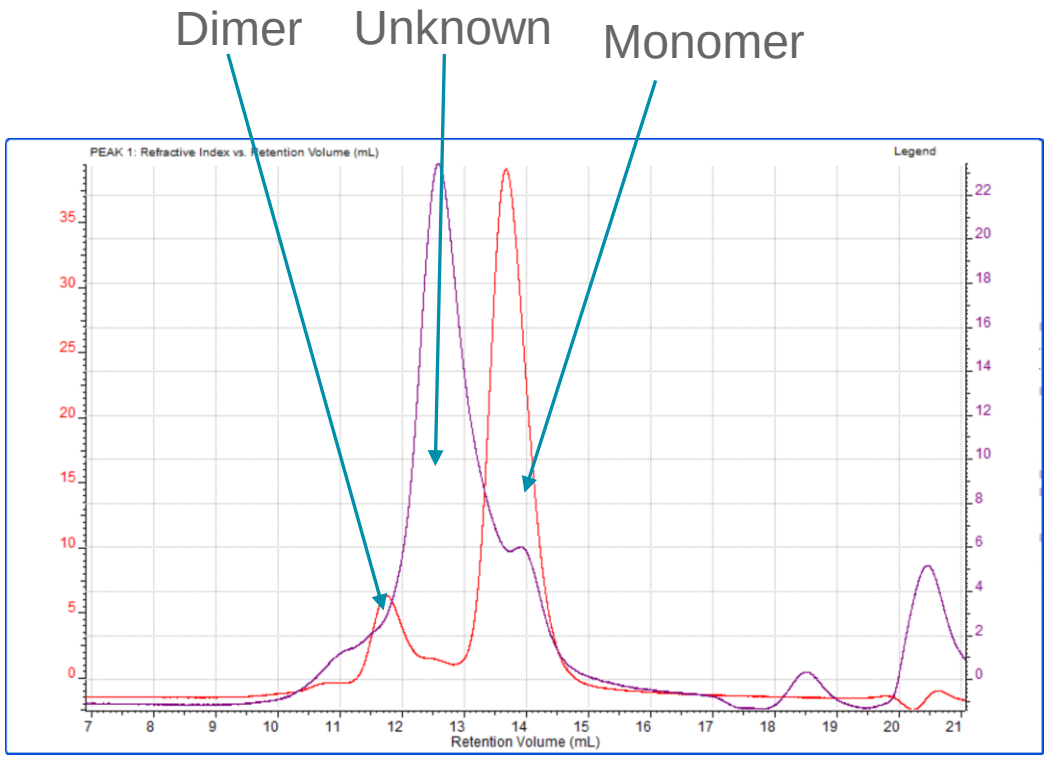


Oligomeric state

HSP + ATP

- HSP in the presence of ATP leads to a big shift in the oligomeric state
  - Displays reversible self association





Stoichiometry

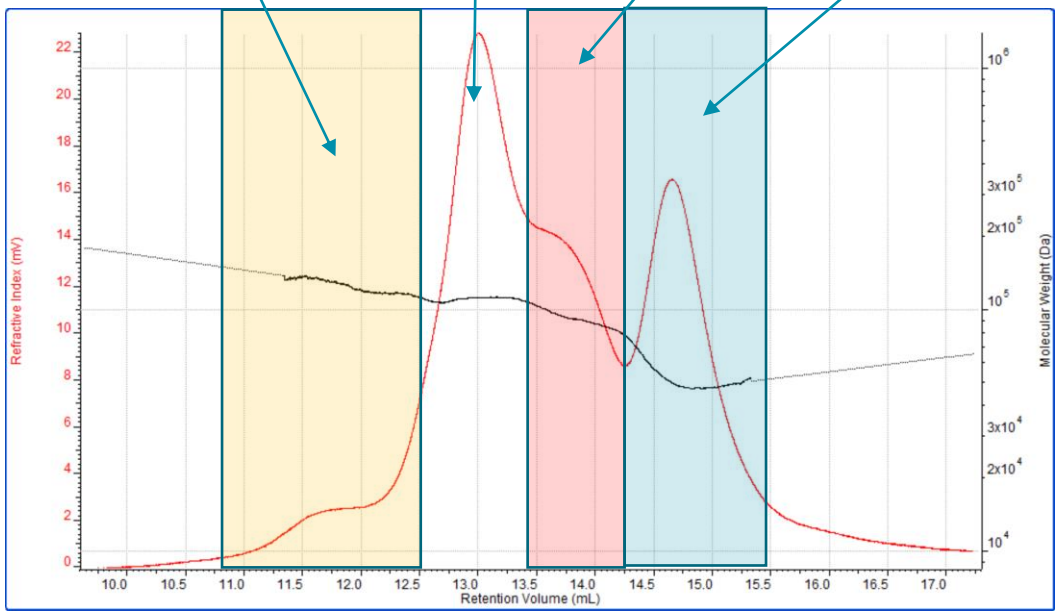
TOM and HSP

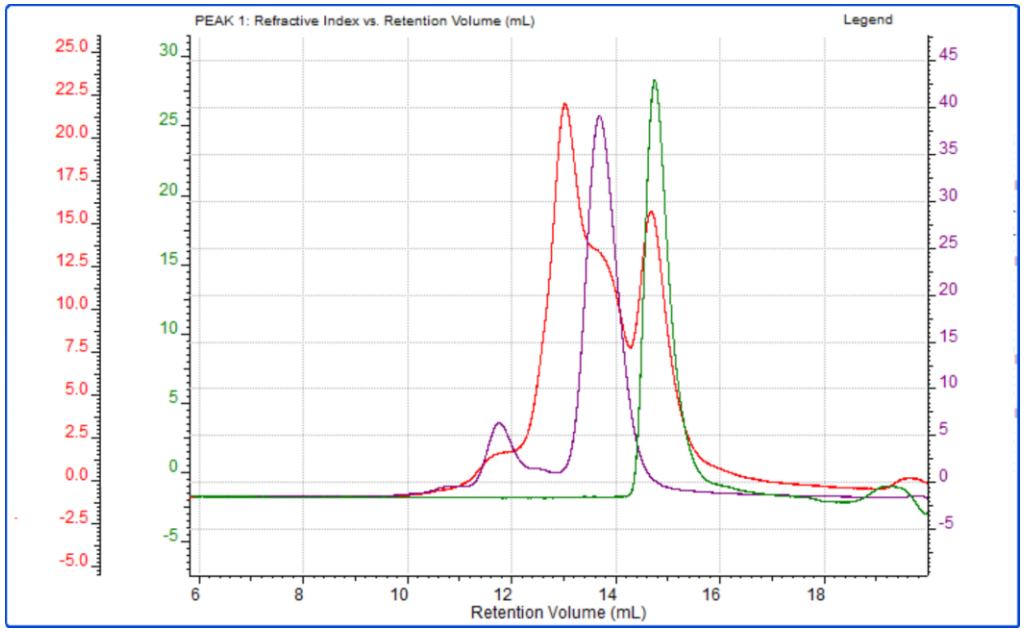
110kDa – HSP-TOM complex – 1:1 ratio

Higher order oligomers

HSP monomer

TOM monomer



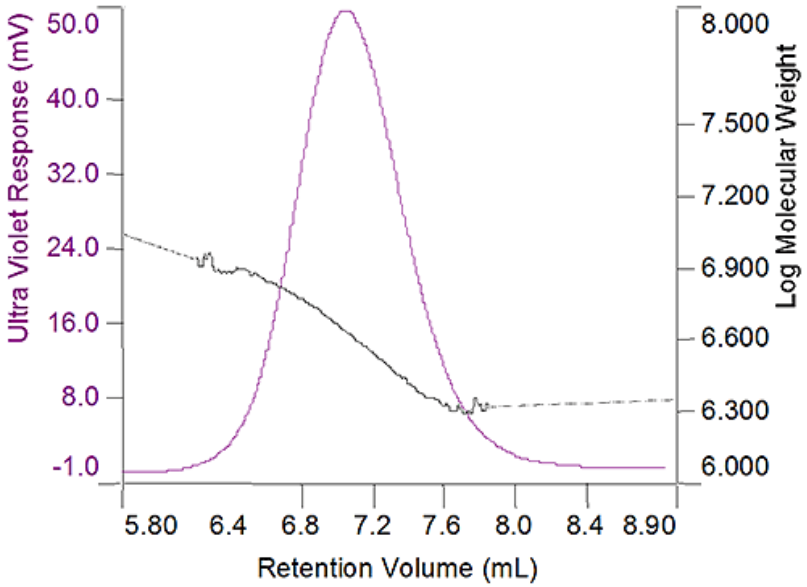


Overlay of Complex, TOM and HSP monomer

Plasmid DNA

- Recombinant plasmid DNAs are used as both raw materials and active ingredients in DNA vaccines.
- DNA is a very long linear molecule
  - high IV
  - high molecular weight
- Cant be analysed using MS due to large plasmid size
- Several plasmid DNA vaccines are being developed
  - Three different structures compared
    - Supercoiled
    - Open circle
    - Linear

Plasmid DNA



Sample	Expected Mw (MDa)	Calculated Mw (MDa)	IV (dL/g)	Rh (nm)
DNA1 – SC	3.86	4.16	5.3	66
DNA1 – Lin		3.75	14.2	87
DNA1 – OC		3.69	10.8	89
DNA2 – SC	4.22	4.30	4.8	64
DNA2 – Lin		4.17	14.0	89
DNA2 – OC		4.46	12.1	91

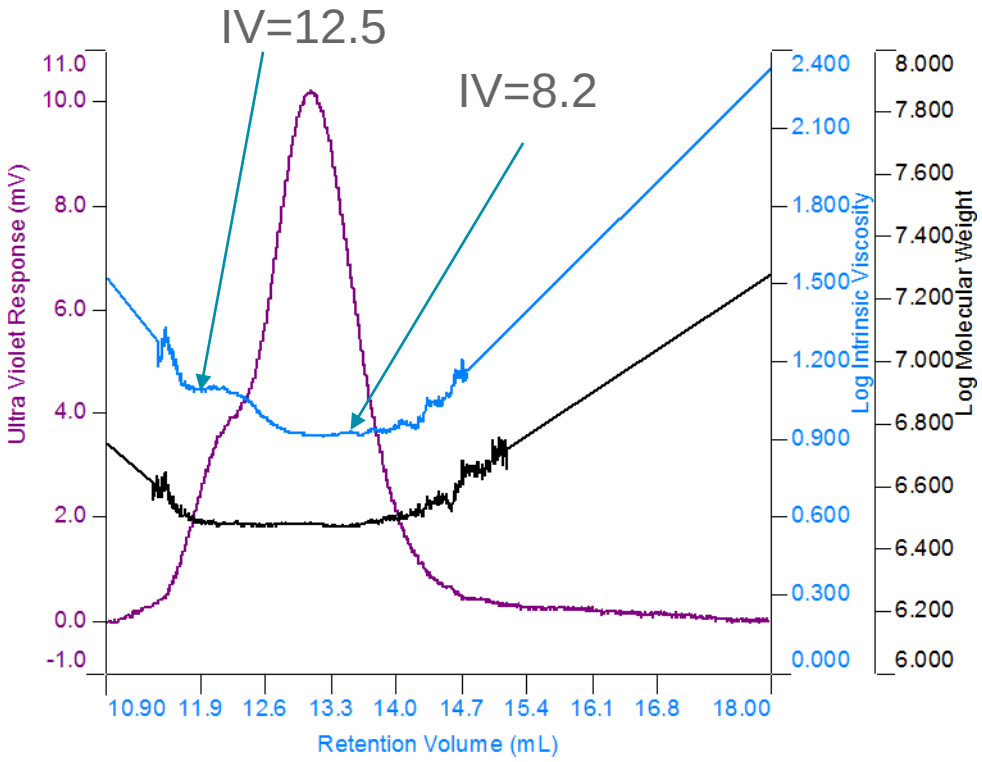
- The hydrodynamic radius data shows that the supercoiled form is smaller in size than the open circle and linear forms of DNA.
- There is also a very small difference in size between the open circle and linear forms of DNA.

Plasmid DNA

DNA 3

Sample	Expected Mw (MDa)	Calculated Mw (MDa)	IV (dL/g)	Rh (nm)
DNA3 – SC	3.2	3.69	4.6	62.8
DNA3 – Lin		3.75	12.2	86.8
DNA3 – OC		3.50	9.1	77.9

- The contaminating shoulder in a sample of DNA can be characterised by IV even though it has the same molecular weight
  - IV of 8.2 is probably open coil DNA
  - IV of 12.5 is probably linear DNA
- Therefore, this open coil sample is either contaminated by linear DNA or some has broken down into linear



Conclusions

- SEC is a great way to compare many different samples
  - Samples from different sources
  - Different protein types
  - Samples from different formulations

- Multi-Detection SEC is an invaluable tool for the assessment of biologicals.
  - Absolute Mw
  - Dispersity (Mw/Mn)
  - Oligomeric state and aggregation
  - Formulation effects, purity, stability, product and process related stress
  - Conjugate analysis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