Use of DLS and SEC-MALS for the characterization of proteins in solution: principle and applications

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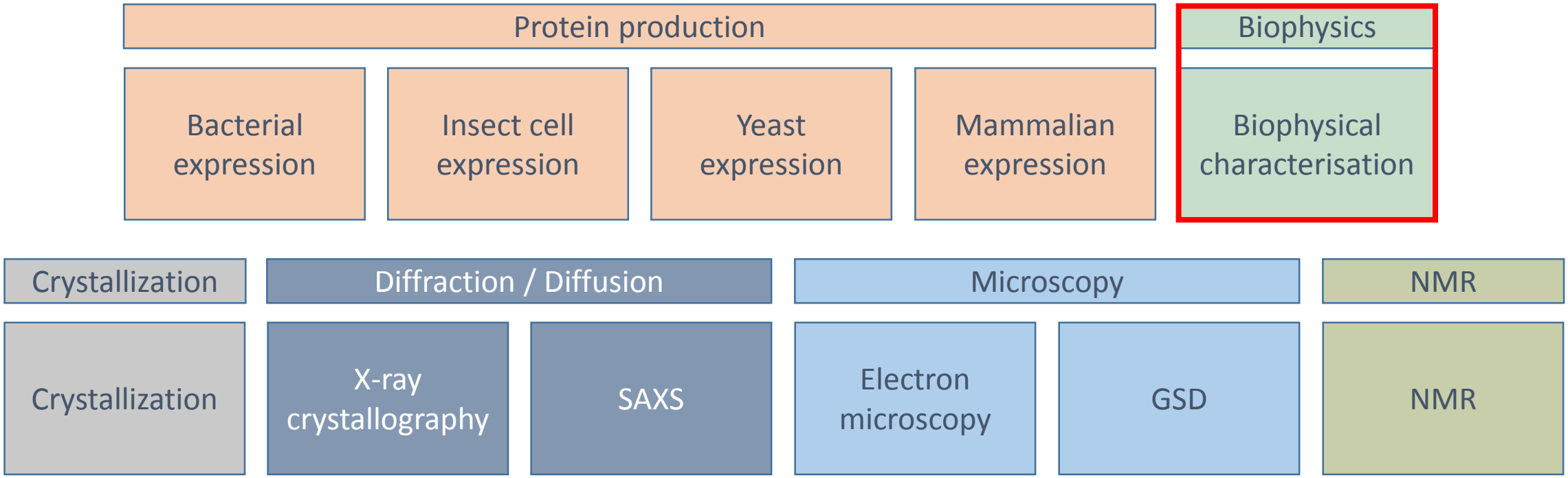
1st P4EU Workshop

November 6th 2019

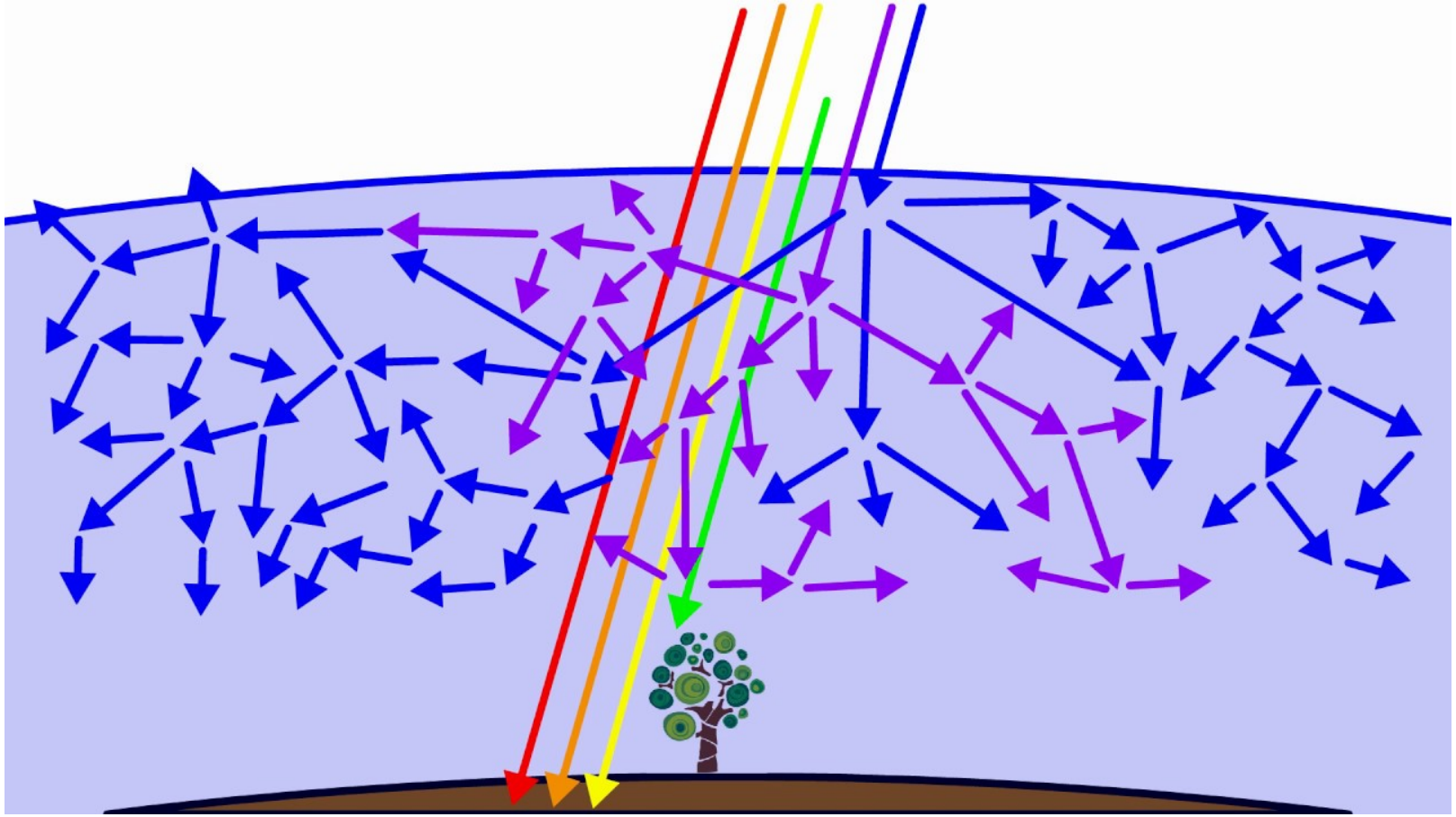
The Integrated Structural Biology Platform



Biophysical characterization :

- Analytical ultra centrifugation
- Isothermal Microcalorimetry
- Circular Dichroism
- Microscale thermophoresis
- DLS
- SEC-MALS
- Nano differential scanning fluorimetry
- Thermal shift assay

Why is the sky blue?



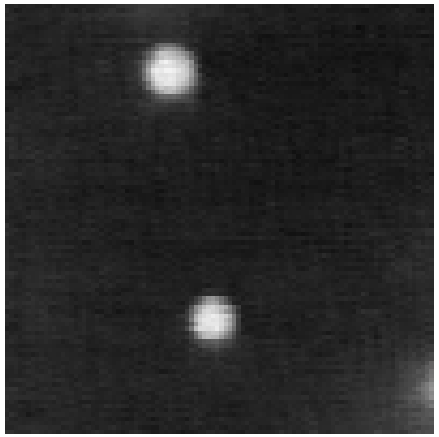
The white light from the sun reaches Earth's atmosphere and is scattered in all directions by all the gases and particles in the air. Blue and violet are scattered more than other colors because they have shorter and smaller wavelengths.

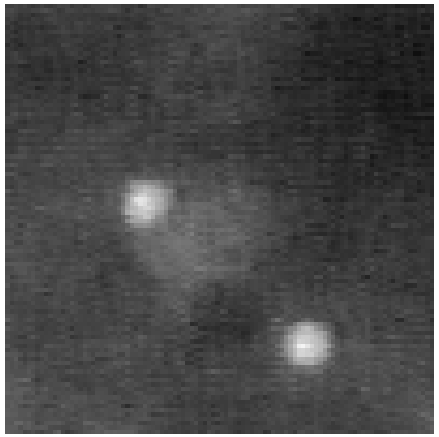
From Scientificprotocols

Static light scattering VS Dynamic light scattering

Static Light Scattering ⇨ overall intensity of scattered light (over a “long time”)

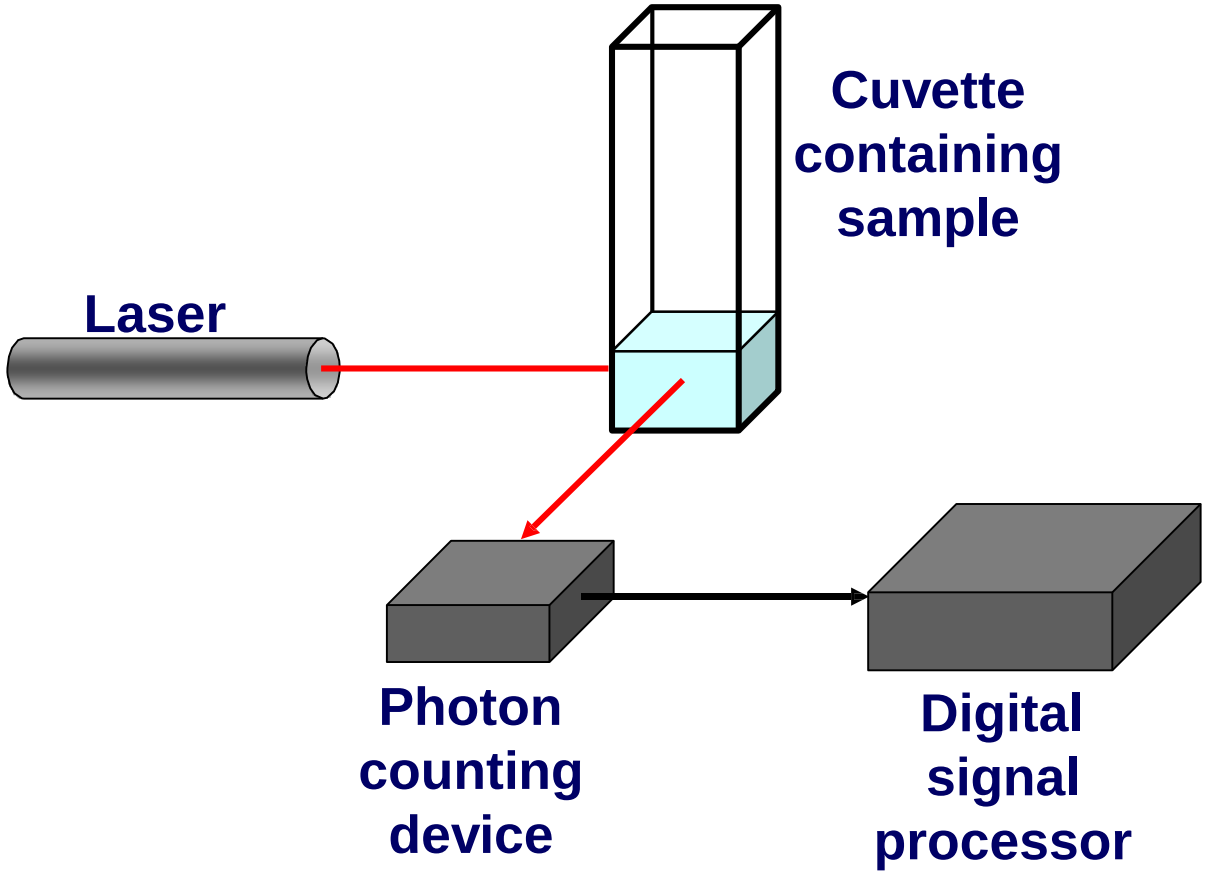
Dynamic Light Scattering ⇨ time dependent fluctuations in the intensity of the scattered light (over a short time) caused by random motion of the macromolecules in the solution





Dynamic Light Scattering (DLS)

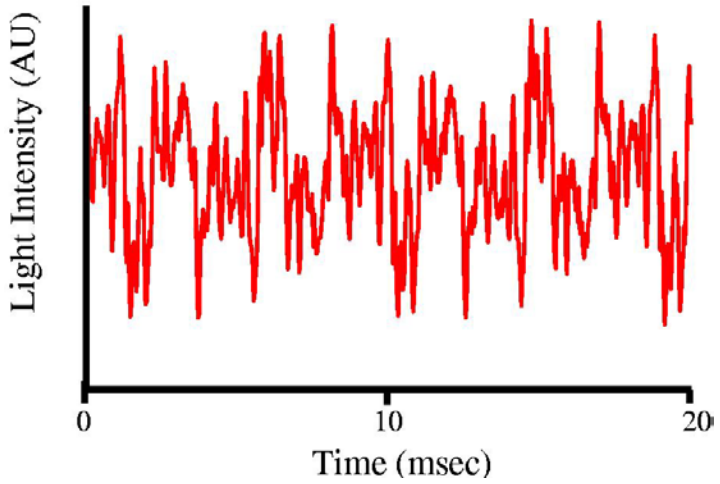
Dynamic light scattering



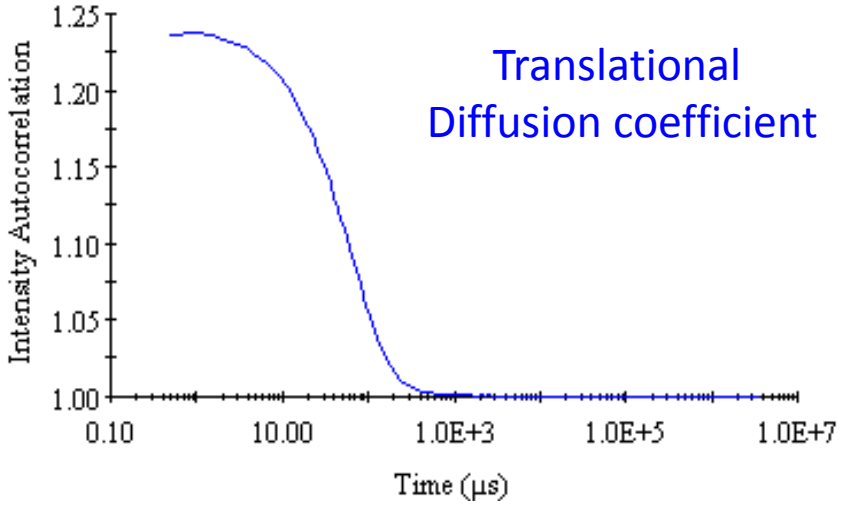
From Malvern Panalytical

Dynamic light scattering

Light Intensity Fluctuations

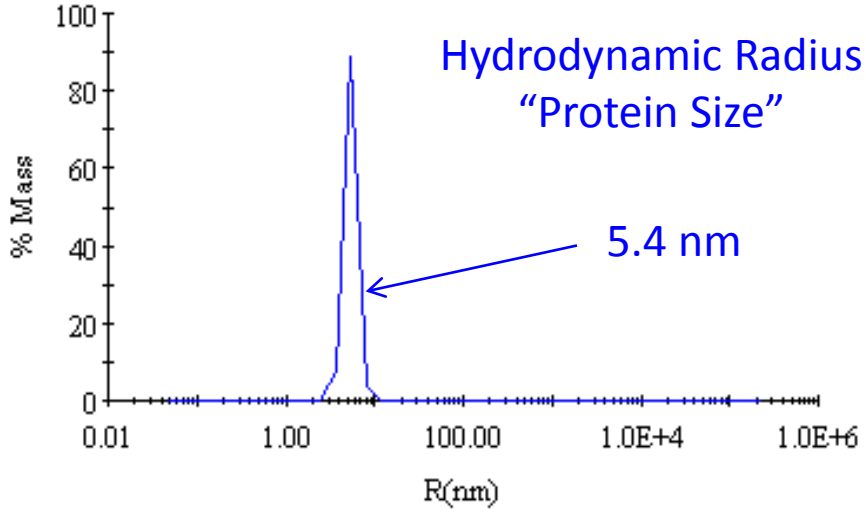


Autocorrelation Analysis



Translational Diffusion coefficient

Stokes-Einstein Relationship



Hydrodynamic Radius "Protein Size"

5.4 nm

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Dynamic light scattering

Non invasive technique for measuring the size of molecules in suspension

DLS measures the speed of particules undergoing Brownian motion:

- small particules diffuse rapidly
- large particules diffuse slowly

Velocity of the Brownian motion is defined by the translational diffusion coefficient Dt.

The motion is random, but related to: particule size, sample viscosity, temperature and sample shape.

Dynamic light scattering

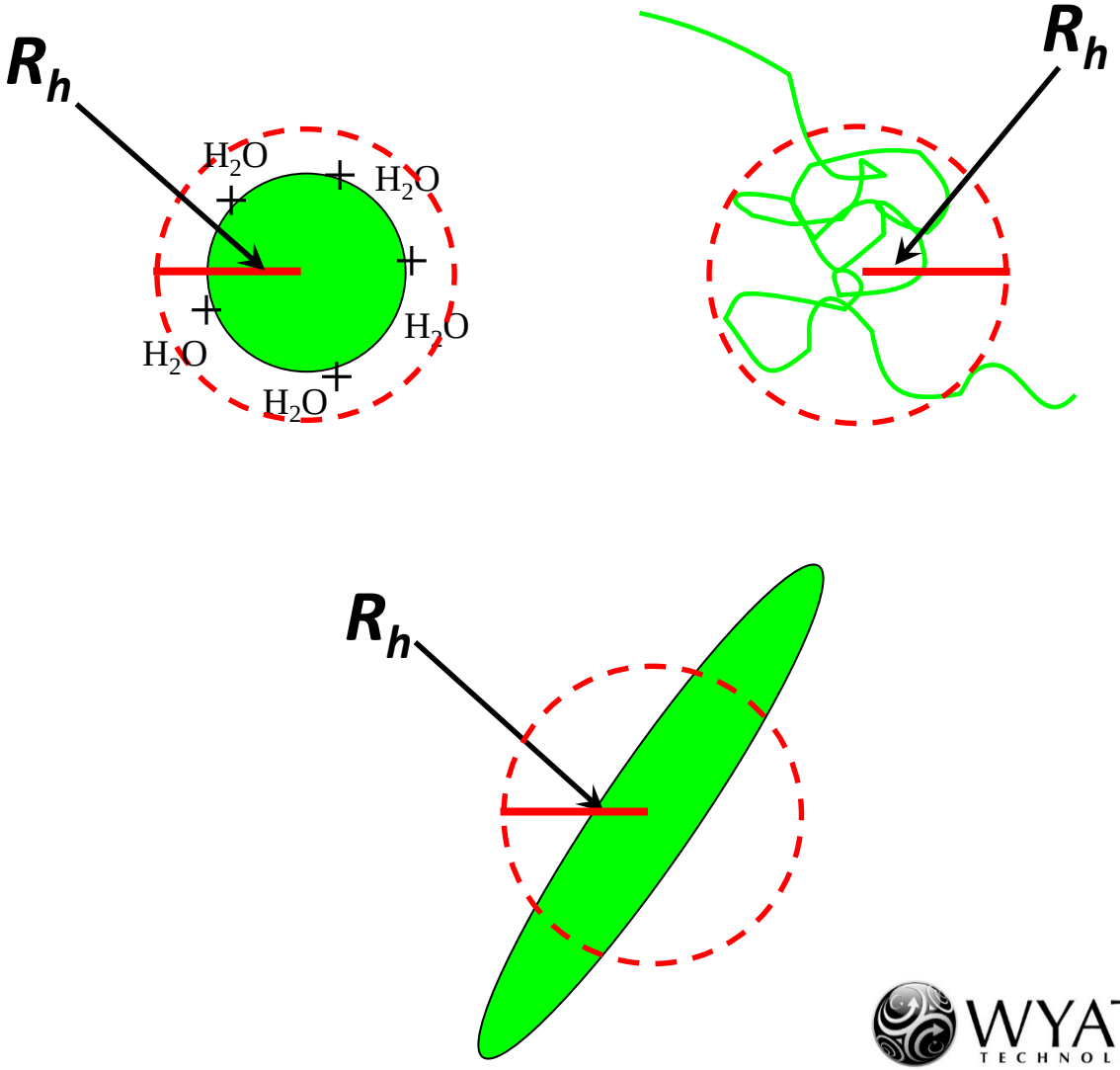
Stokes - Einstein relationship

$$D_t = \frac{kT}{6\pi\eta R_h}$$

k_B – Boltzmann's constant
T – temperature (Kelvin)
η – viscosity of solvent
R_h – hydrodynamic radius

What is R_h?

R_h or Hydrodynamic Radius :
radius of a sphere that diffuses at the same speed as "our" sample



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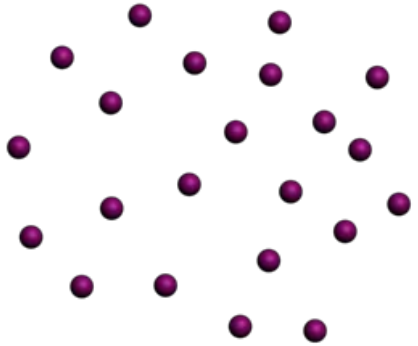
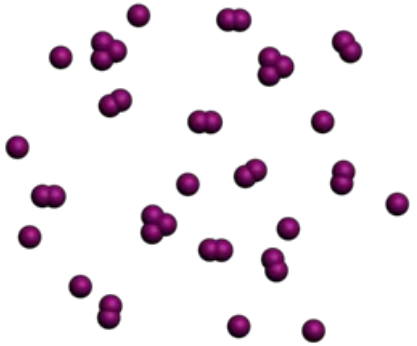
Analyzing Particle Size Distributions

Monomodal

Monodisperse

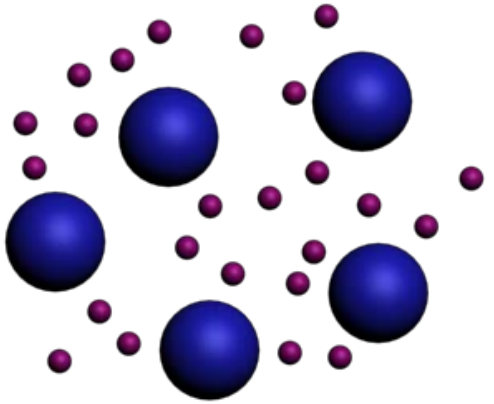


Polydisperse

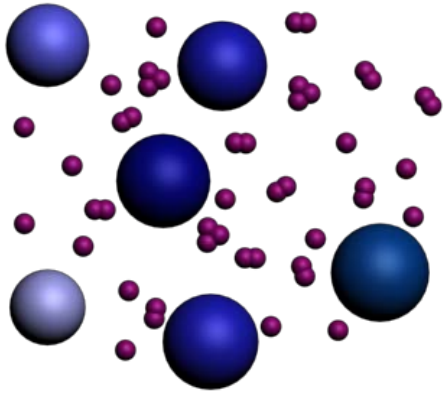


Multimodal

Monodisperse



Polydisperse



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Analyzing Particle Size Distributions

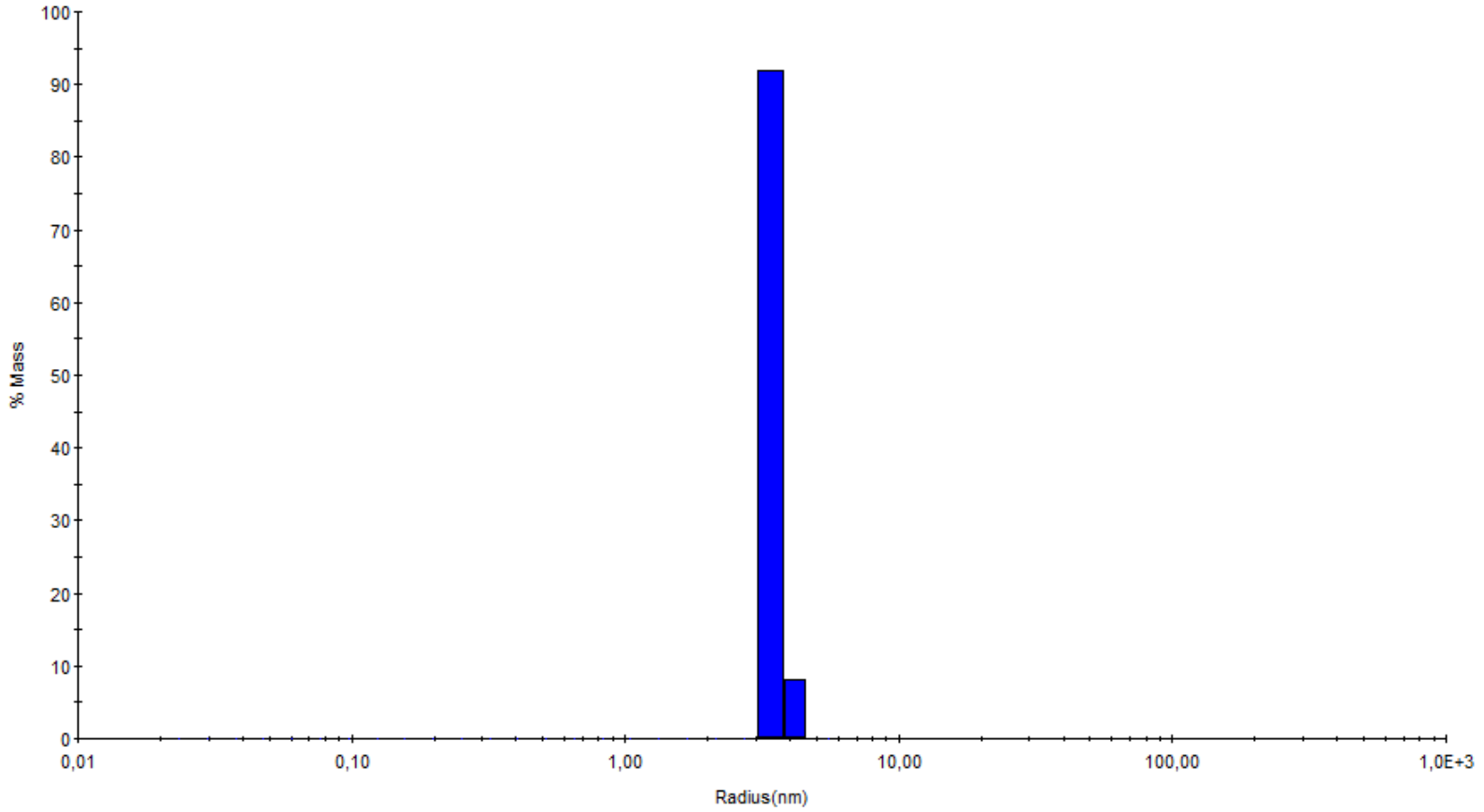
Example of monomodal and monodisperse sample

MW = 66,2 kDa

R_h = 3,51 nm

Polydispersity = 7,1 %

Estimated Mw = 64 kDa



Analyzing Particle Size Distributions

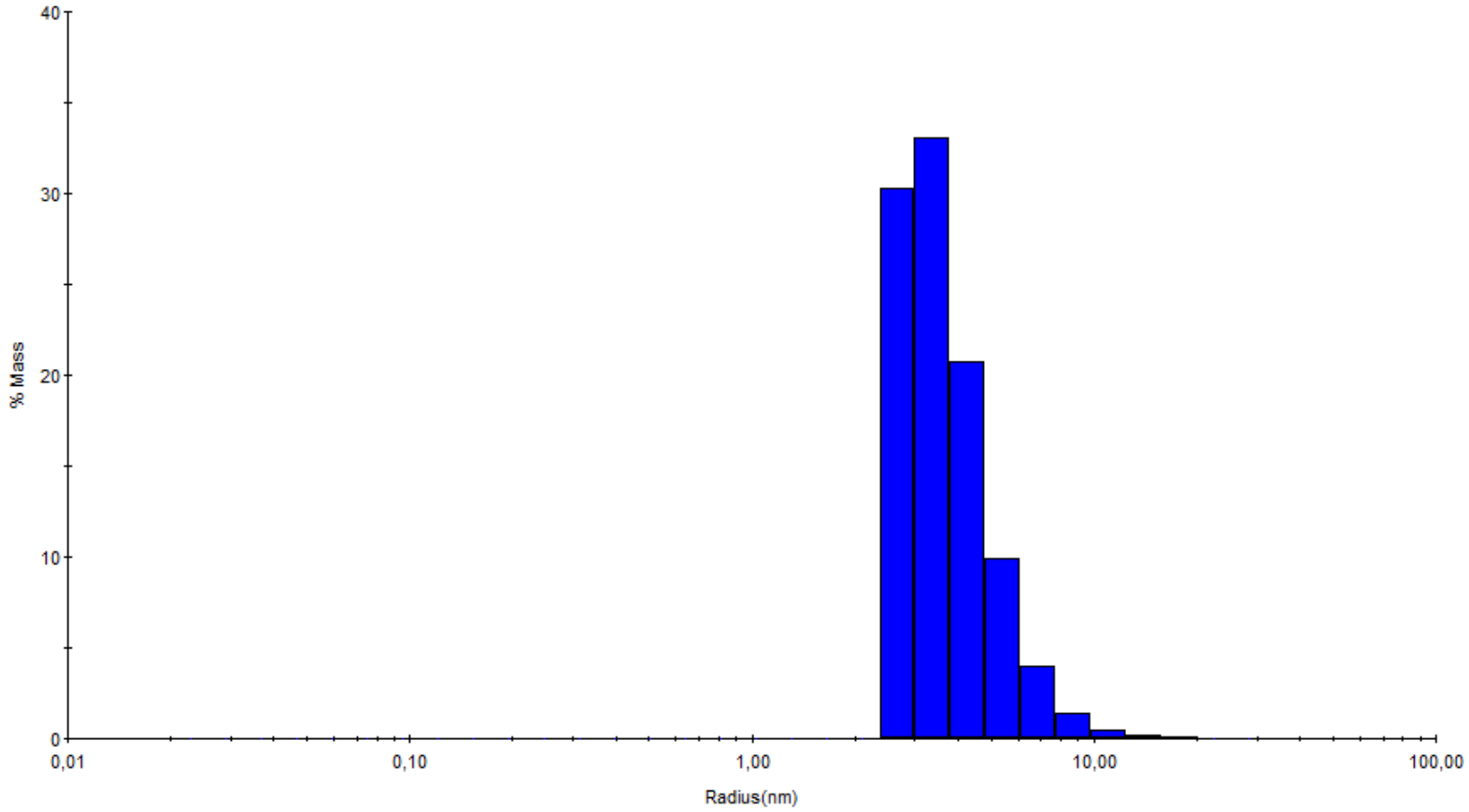
Example of monomodal and polydisperse sample

Mw = 58,3 kDa

R_h = 3,82 nm

Polydispersity = 37,2 %

Estimated Mw = 77 kDa



Analyzing Particle Size Distributions

Example of multimodal and monodisperse sample

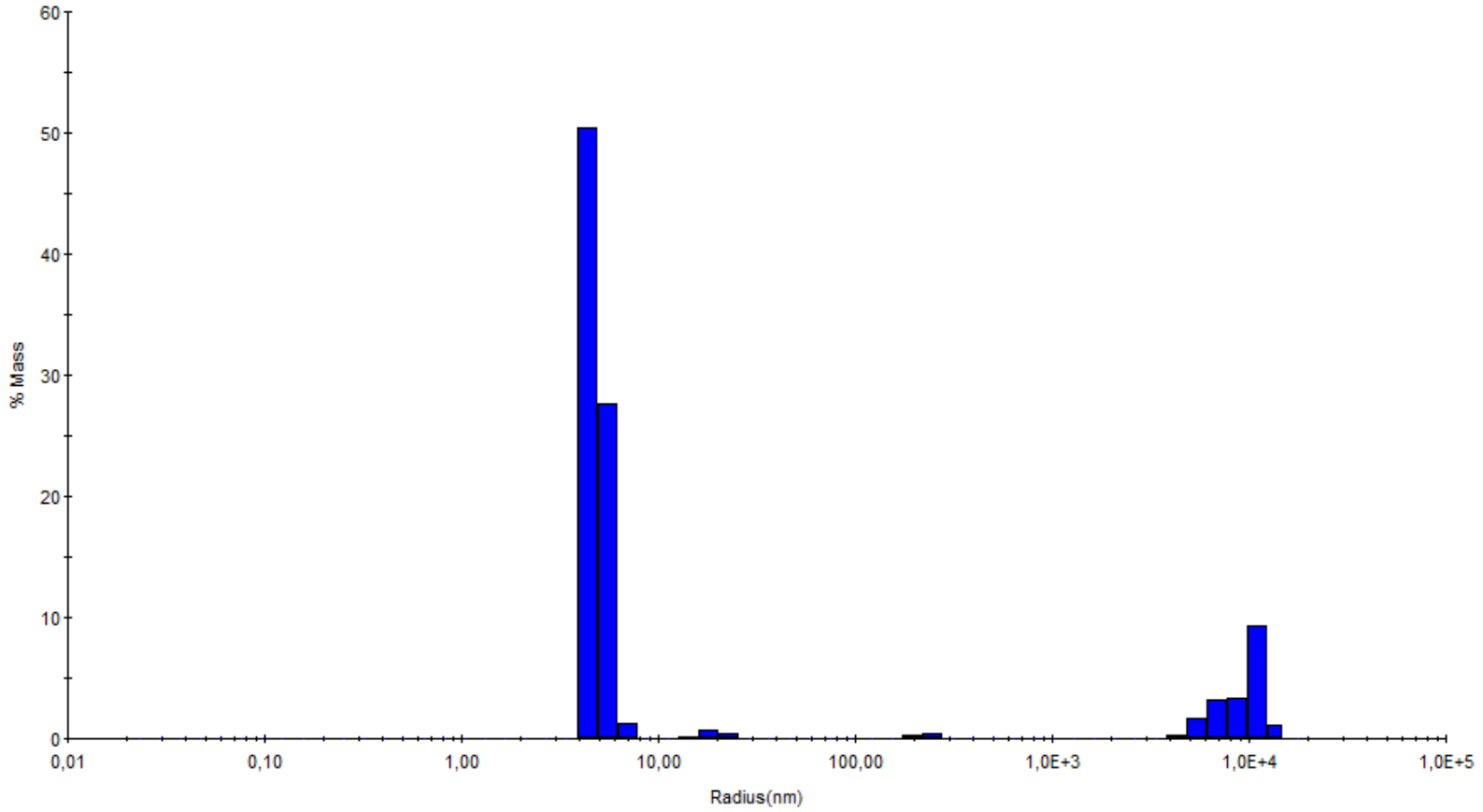
Mw = 58,3 kDa

R_h = 4,80 nm

Polydispersity = 12,8 %

Estimated Mw = 131 kDa

79,2 % of the total mass



Analyzing Particle Size Distributions

Example of multimodal and polydisperse sample

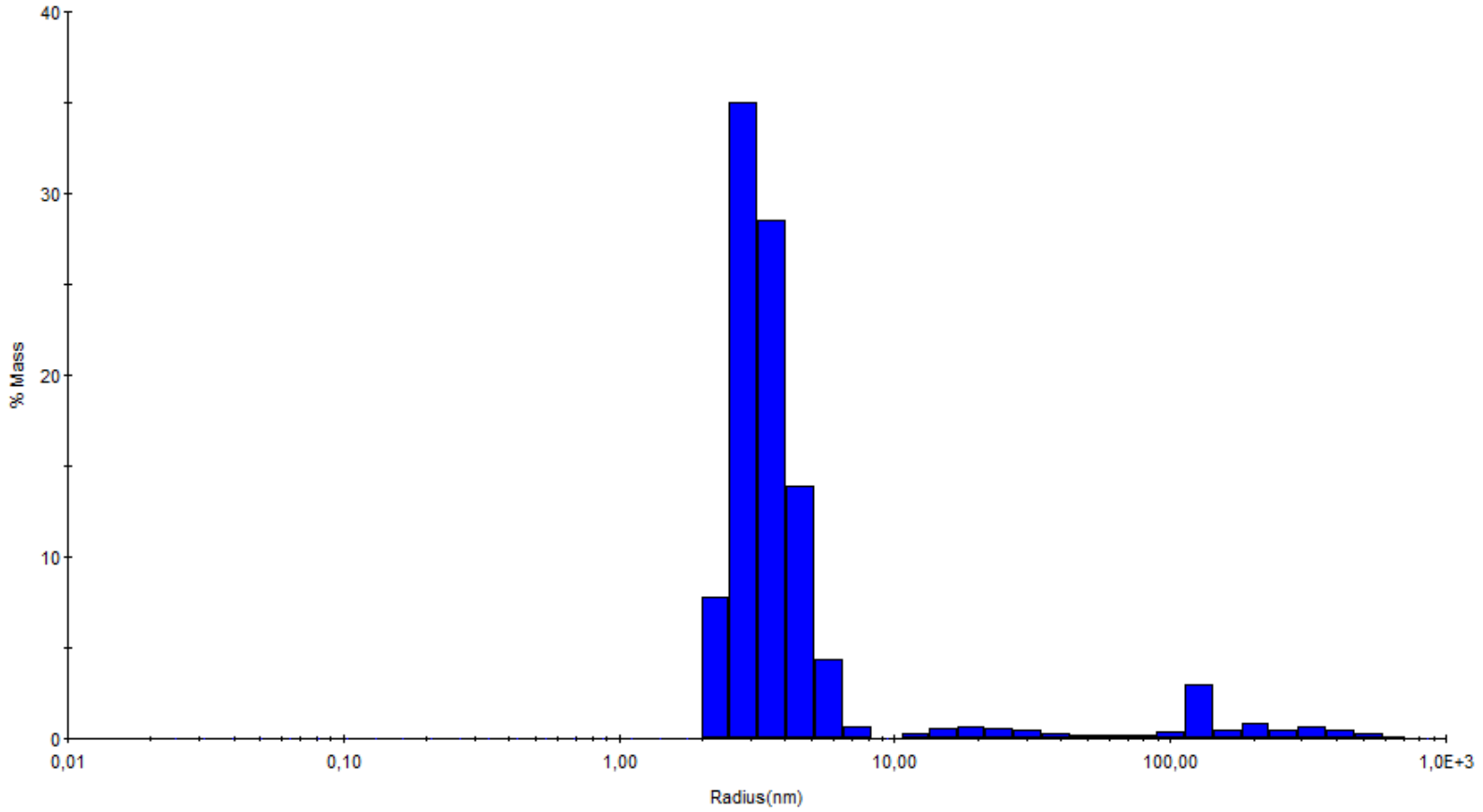
Mw = 32,4 kDa

R_h = 3,50 nm

Polydispersity = 26,5 %

Estimated Mw = 63 kDa

90,1 % of the total mass



Application : is my complex formed?

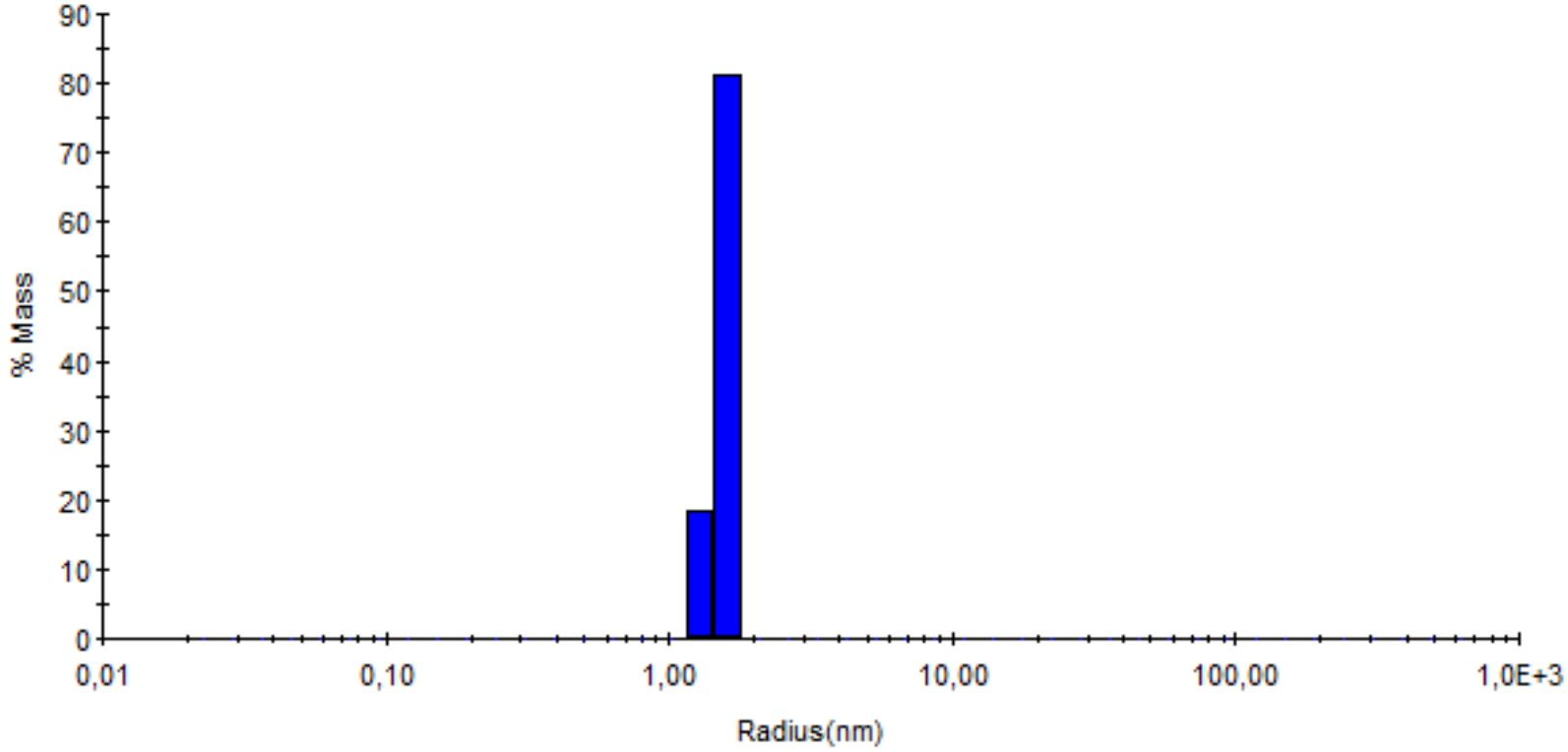
DNA Binding Protein 1

Mw = 10,6 kDa

R_h = 1,59 nm

Polydispersity = 8,5 %

Estimated Mw = 10 kDa



Application : is my complex formed?

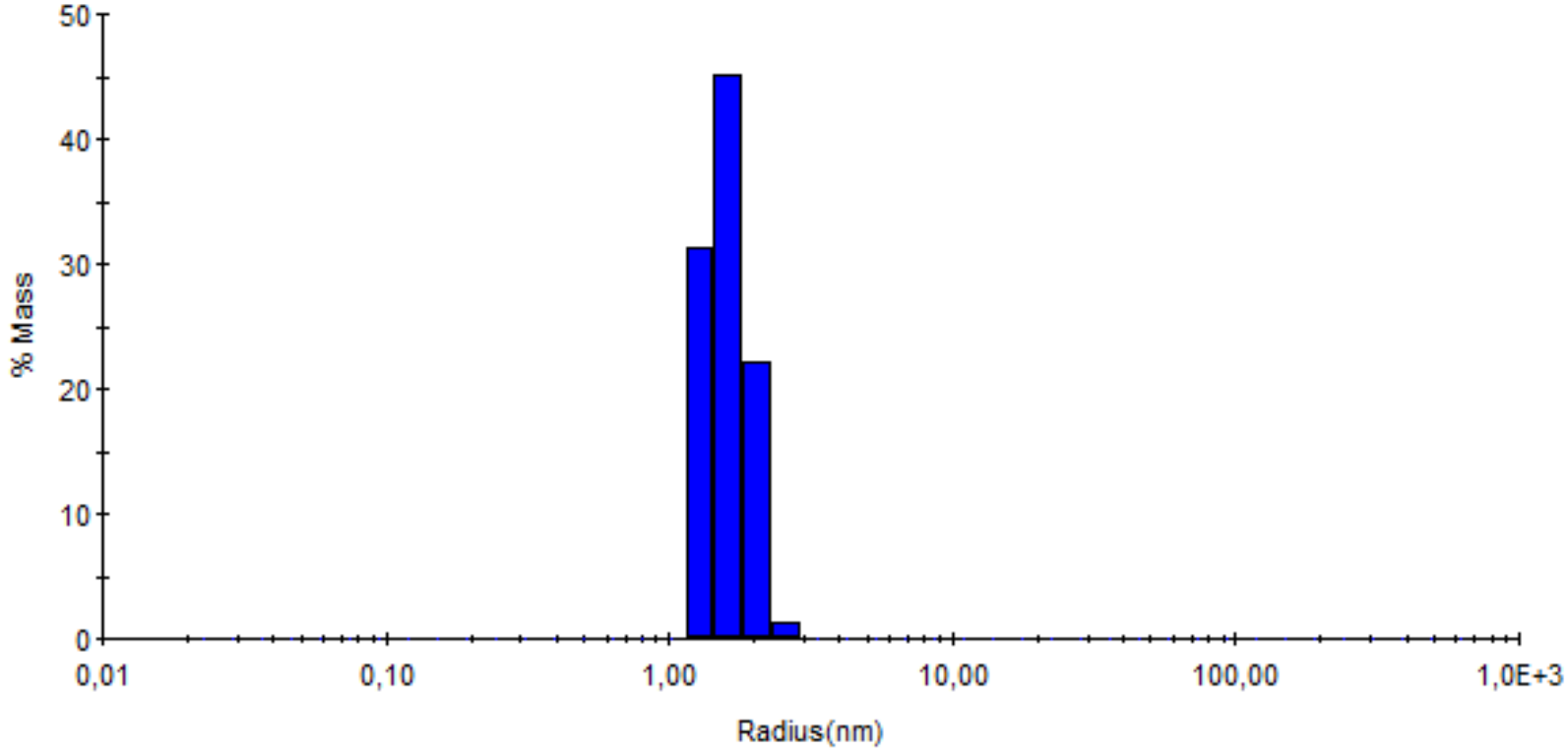
DNA Binding Protein 2

Mw = 10,2 kDa

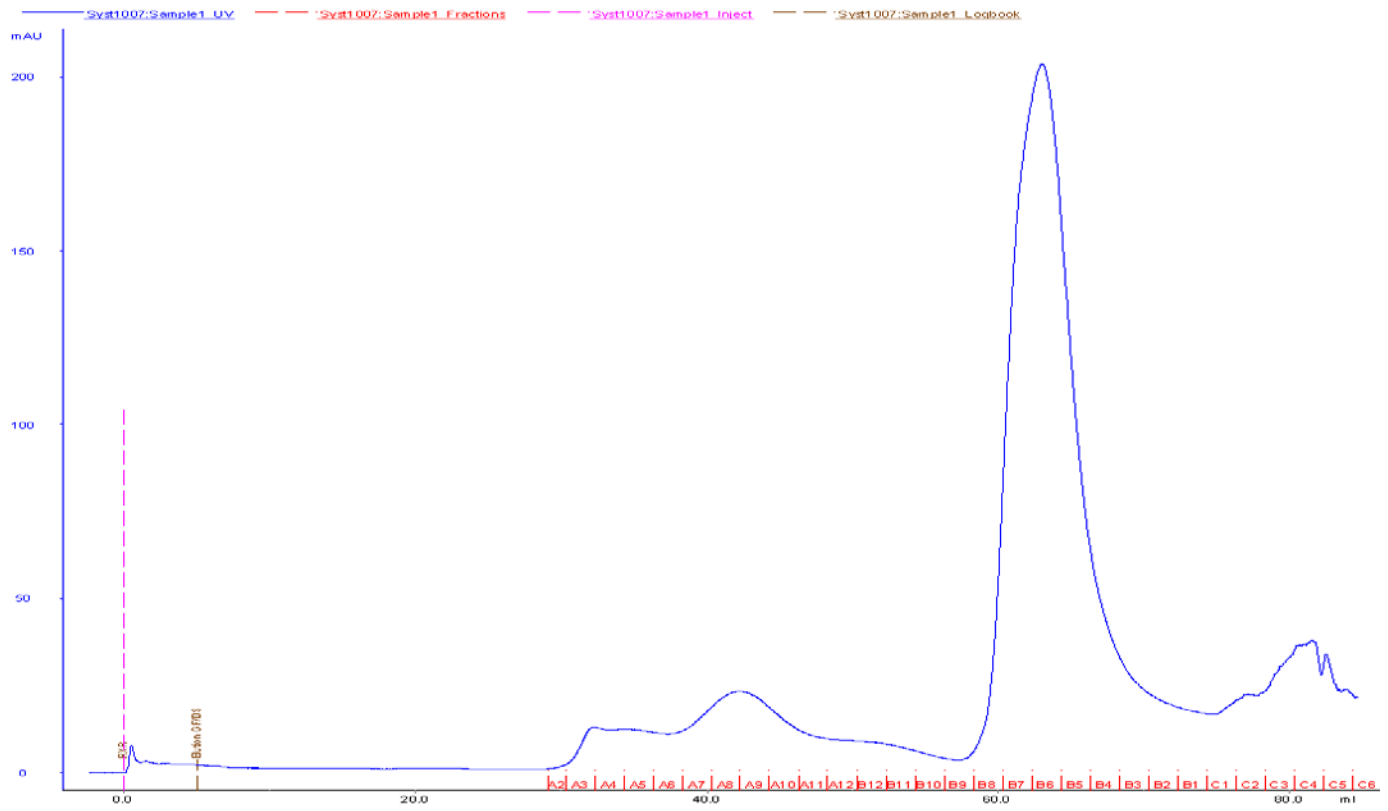
R_h = 1,65 nm

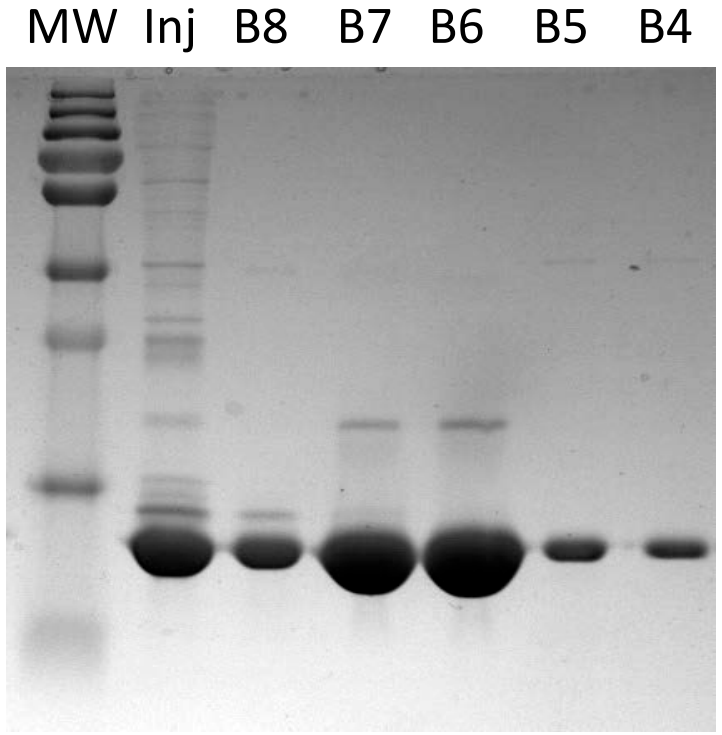
Polydispersity = 18,6 %

Estimated Mw = 11 kDa



Application : selection of chromatography fractions





Fraction	Rh	% Pd	% Mass
B8	3.37	0	87
B7	1.74	19.6	99.6
B6	1.65	18.6	99.8
B5	1.65	2.6	80.6
B4	4.84	11,8	37

Application : is my complex formed?

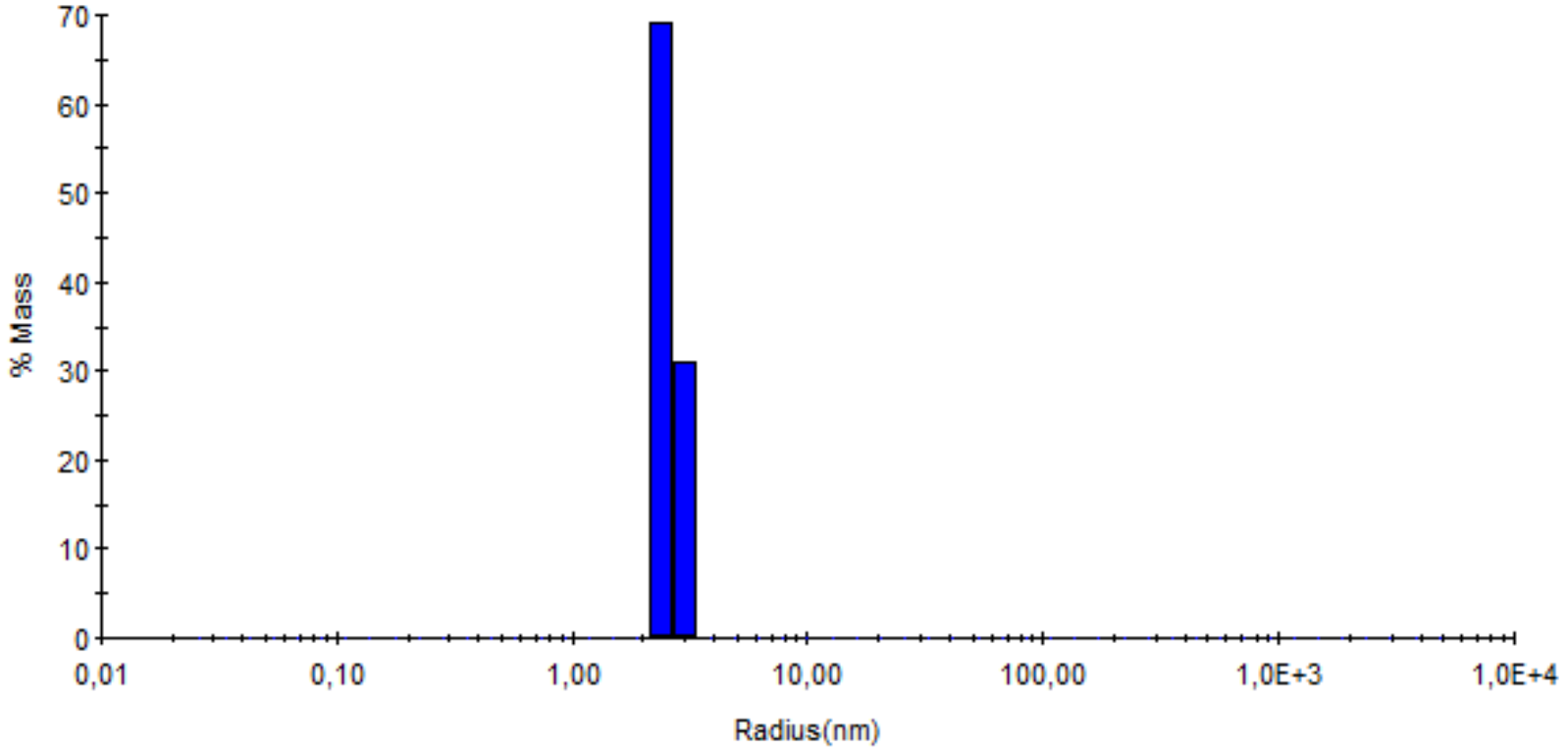
Heterodimer + 16 bp DNA

Mw = 31,4 kDa

R_h = 2,71 nm

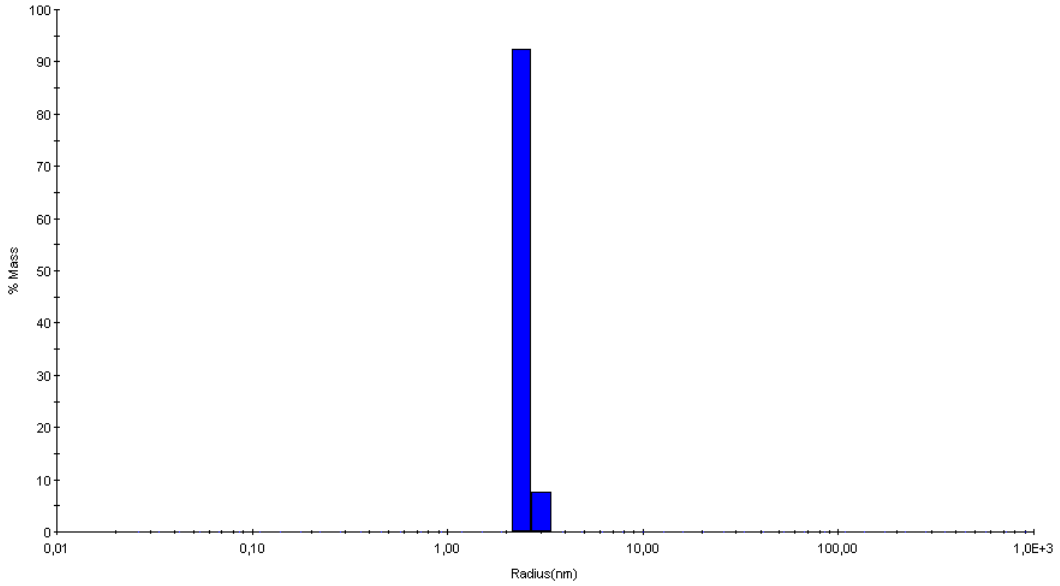
Polydispersity = 11,4 %

Estimated Mw = 32 kDa



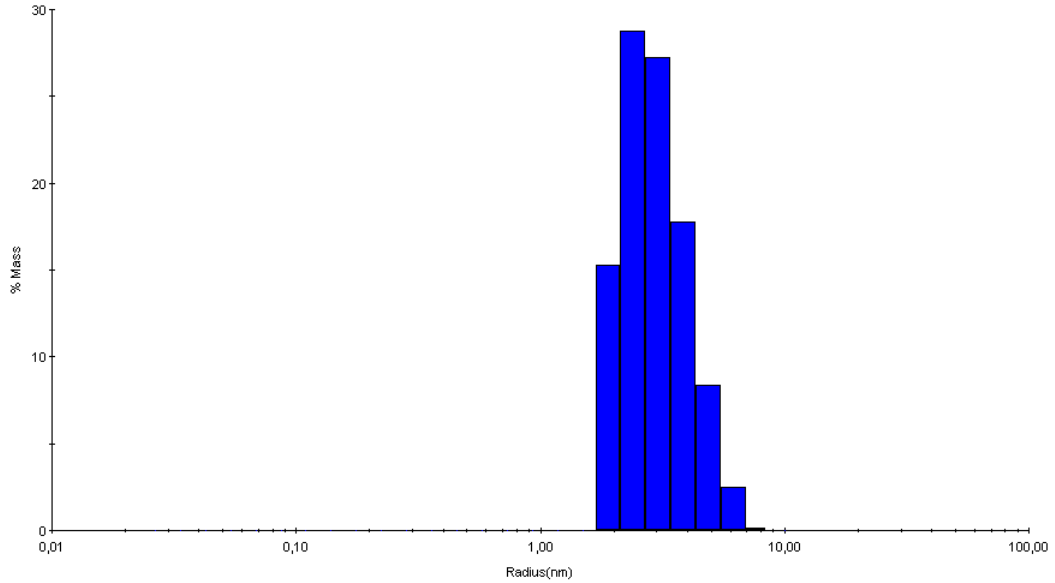
Application : selection of the best DNA

DNA Binding Proteins + DNA 1



Rh = 2,45 nm
Polydispersity = 7,0%

DNA Binding Proteins + DNA 2



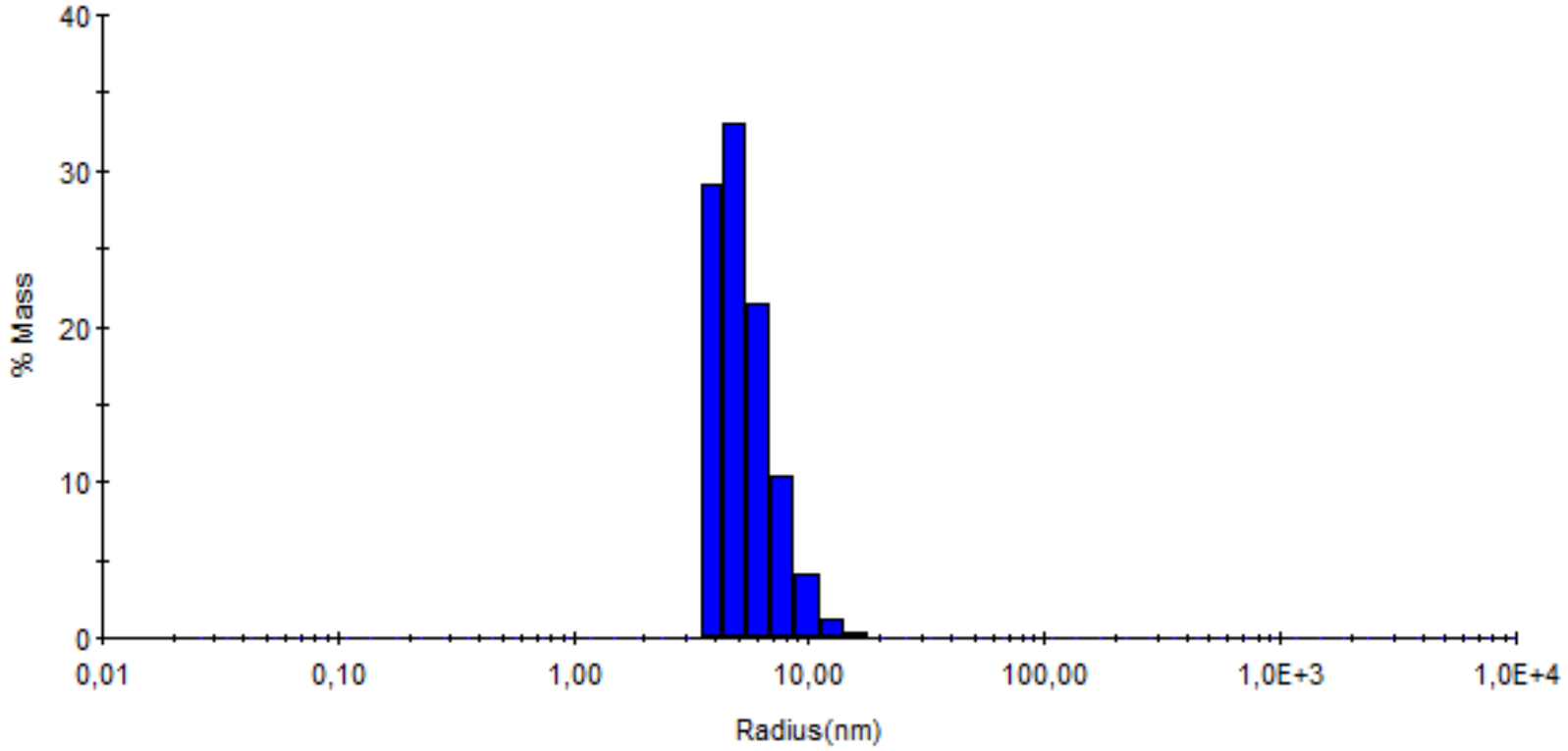
Rh = 3,01 nm
Polydispersity = 32,6%

Application : improvement of purification protocol

2 step purification : affinity + gel filtration

R_h = 5,53 nm

Polydispersity = 33,3 %

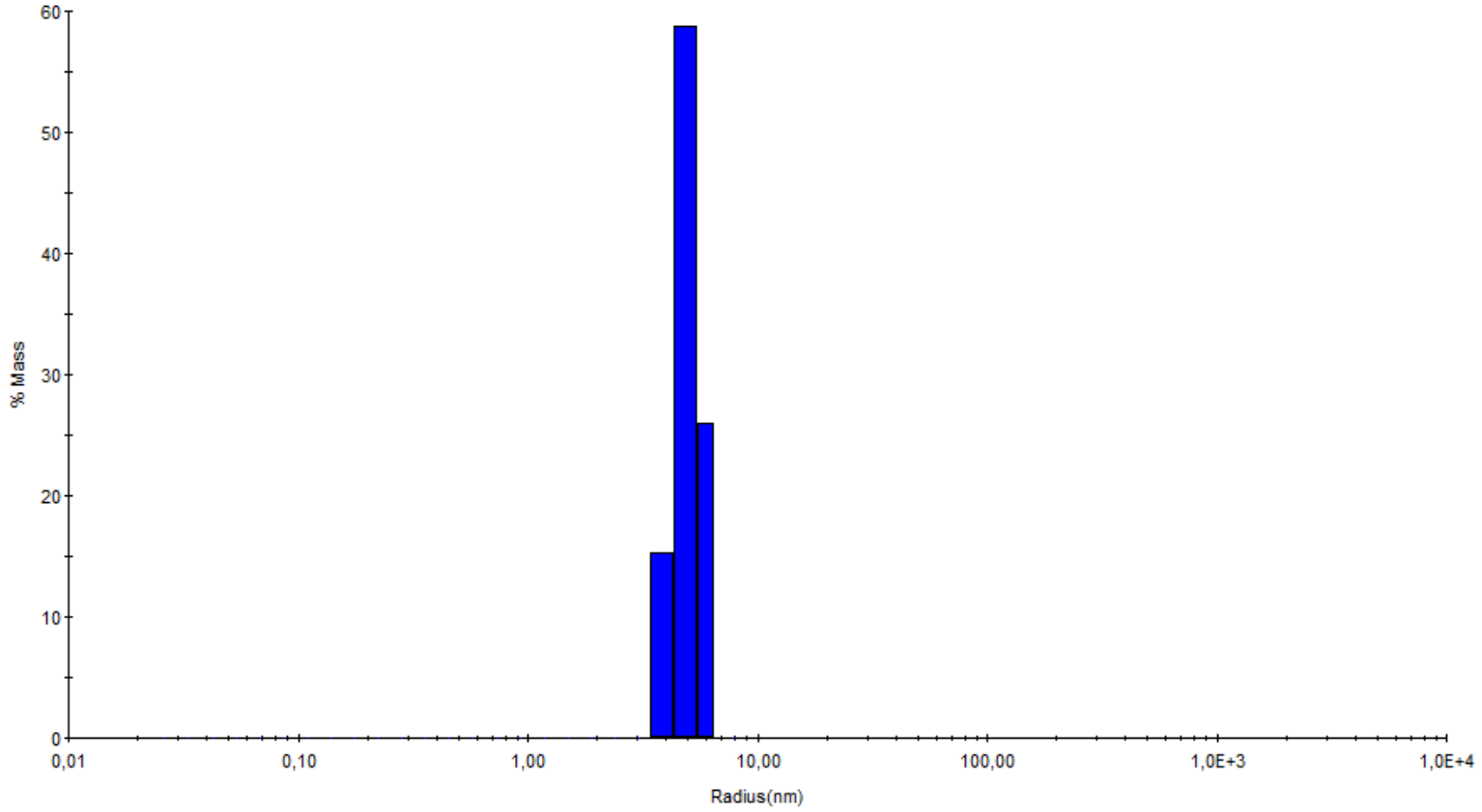


Application : improvement of purification protocol

3 step purification : affinity + ion exchange + gel filtration

R_h = 5,10 nm

Polydispersity = 15,0 %



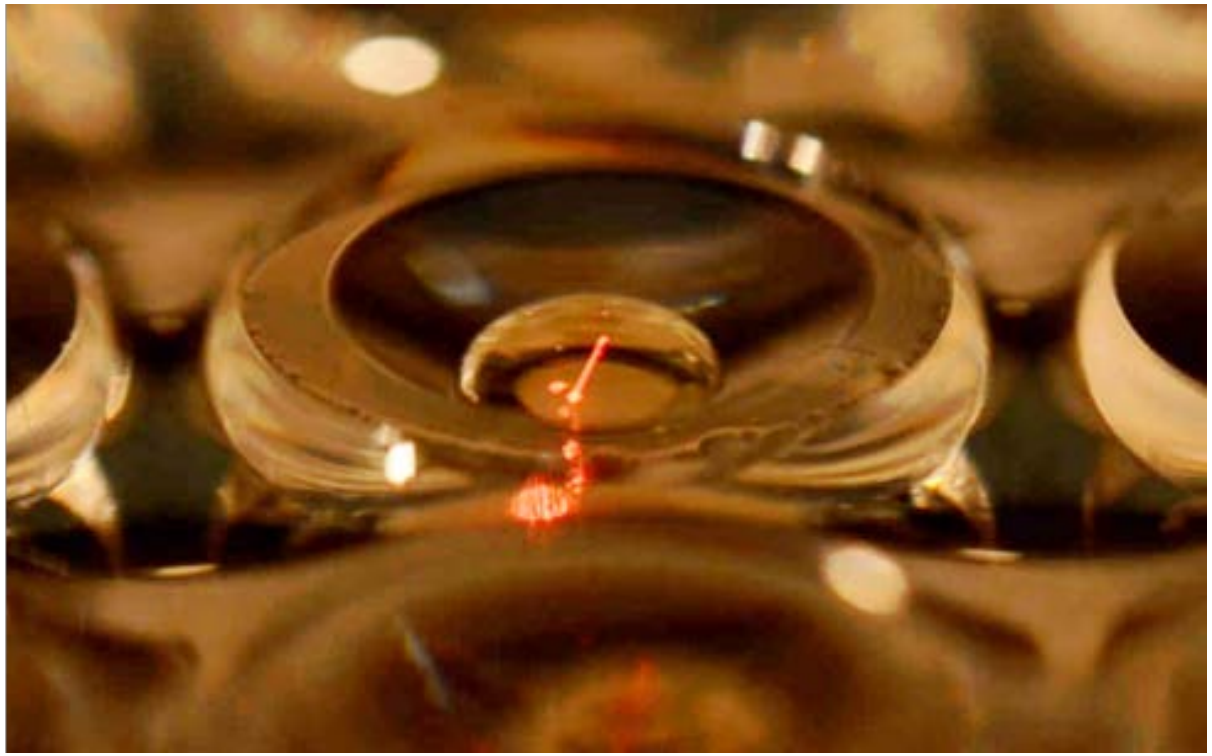
More applications

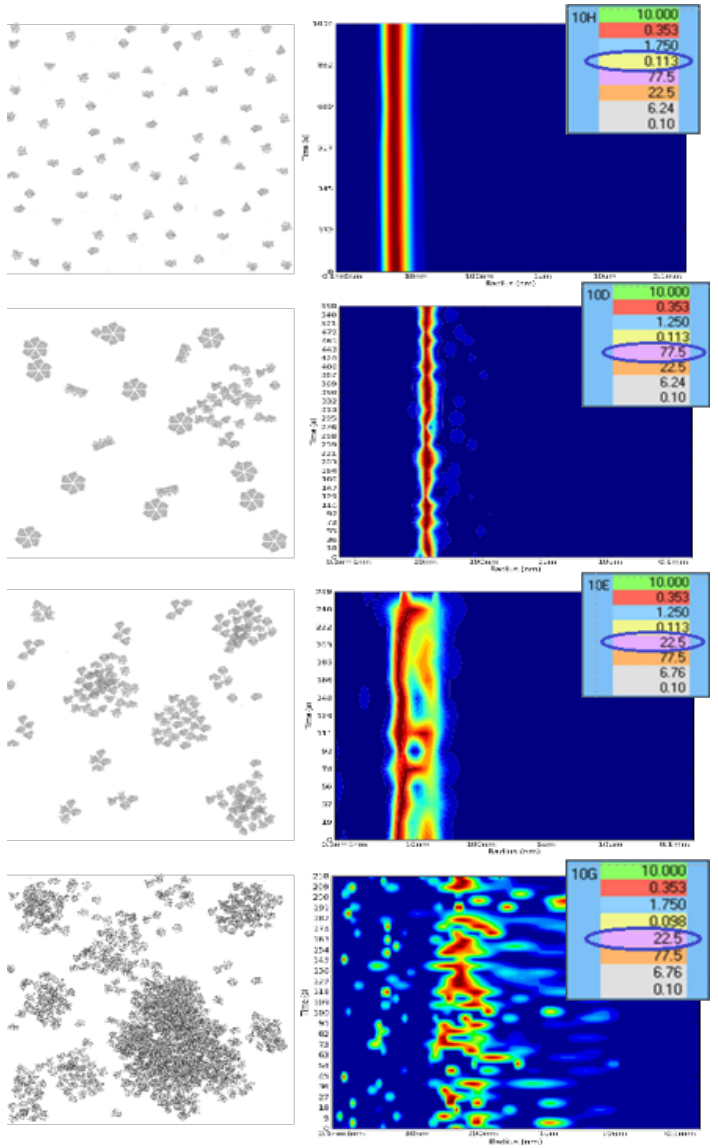
Characterization of biomolecules for further sample evaluation like crystallization, cryo-EM, NMR or SAXS.
e.g. Optimum Solubility Screen, Solubility and Stability Screen…

Condition Screening
After purification, a sample might not be in the desired aggregation state or complex has not formed yet. One strategy to obtain biological is to screen different buffer and salt conditions.

In Situ DLS

Characterization of biomolecules directly in the experiment





Xtal Concepts

Conclusion : why using DLS?

- Non invasive measurement
- Fast result
- Requires only small quantities of sample
- Good for assessing homogeneity of the sample
- Good for detecting trace amounts of aggregates

Limitations of DLS

- DLS measurements are very sensitive to temperature and solvent viscosity. Therefore, the temperature must be kept constant and solvent viscosity must be known.
- DLS is a low-resolution method that cannot separate molecules that are closely related (e.g., monomer and dimer).
- Large aggregates, even a very small amount, will affect the measurements.
- The signal from DLS depends on the size and concentration of macromolecules. To obtain reliable measurements, optimization of the protein concentration may be required.

SEC-MALS

What is SEC-MALS?

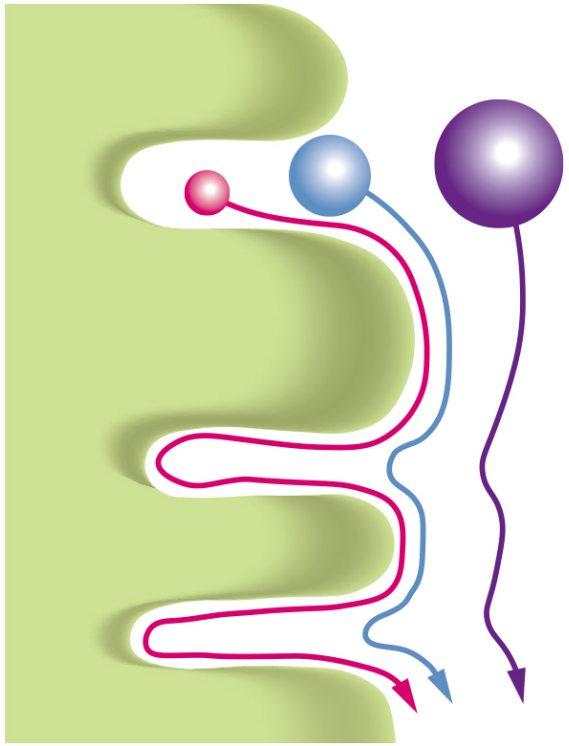
SEC-MALS = Size Exclusion Chromatography coupled with Multi-Angle Light Scattering.

It is dedicated to the determination of the following properties of proteins in solutions:

- Molar mass
- Size
- Aggregation
- Native oligomeric state
- Purity

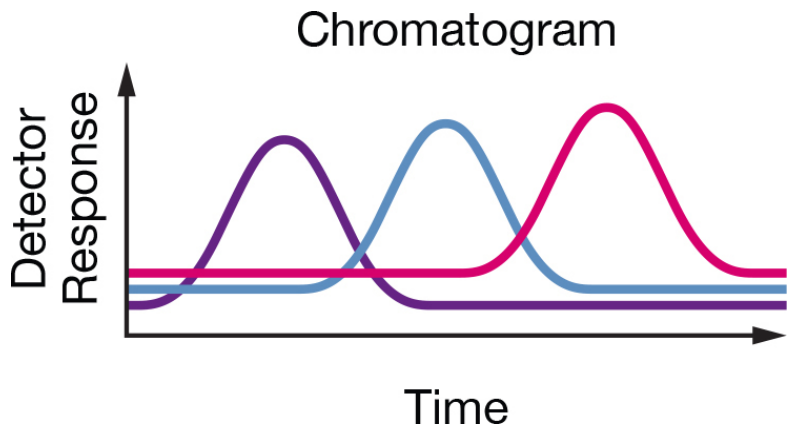
How does conventional SEC works?

Size Exclusion Chromatography (SEC) is a chromatographic method in which molecules are separated based on their size. It means that the elution volume is related to their hydrodynamic volume and not to their molecular weight.



Large particles cannot enter the gel and are excluded. They have less volume to pass through and elute sooner.

Small particles can enter the gel and have more volume to pass trough. They elute later.



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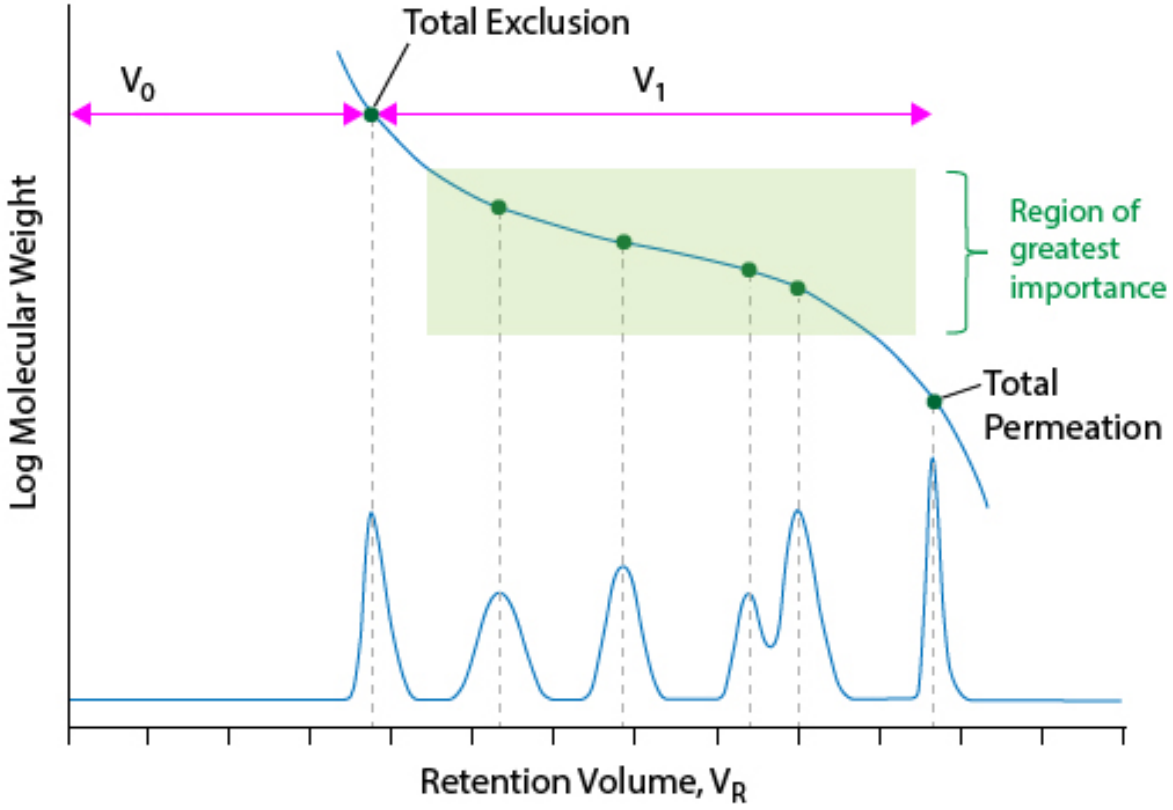
How does conventional SEC works?

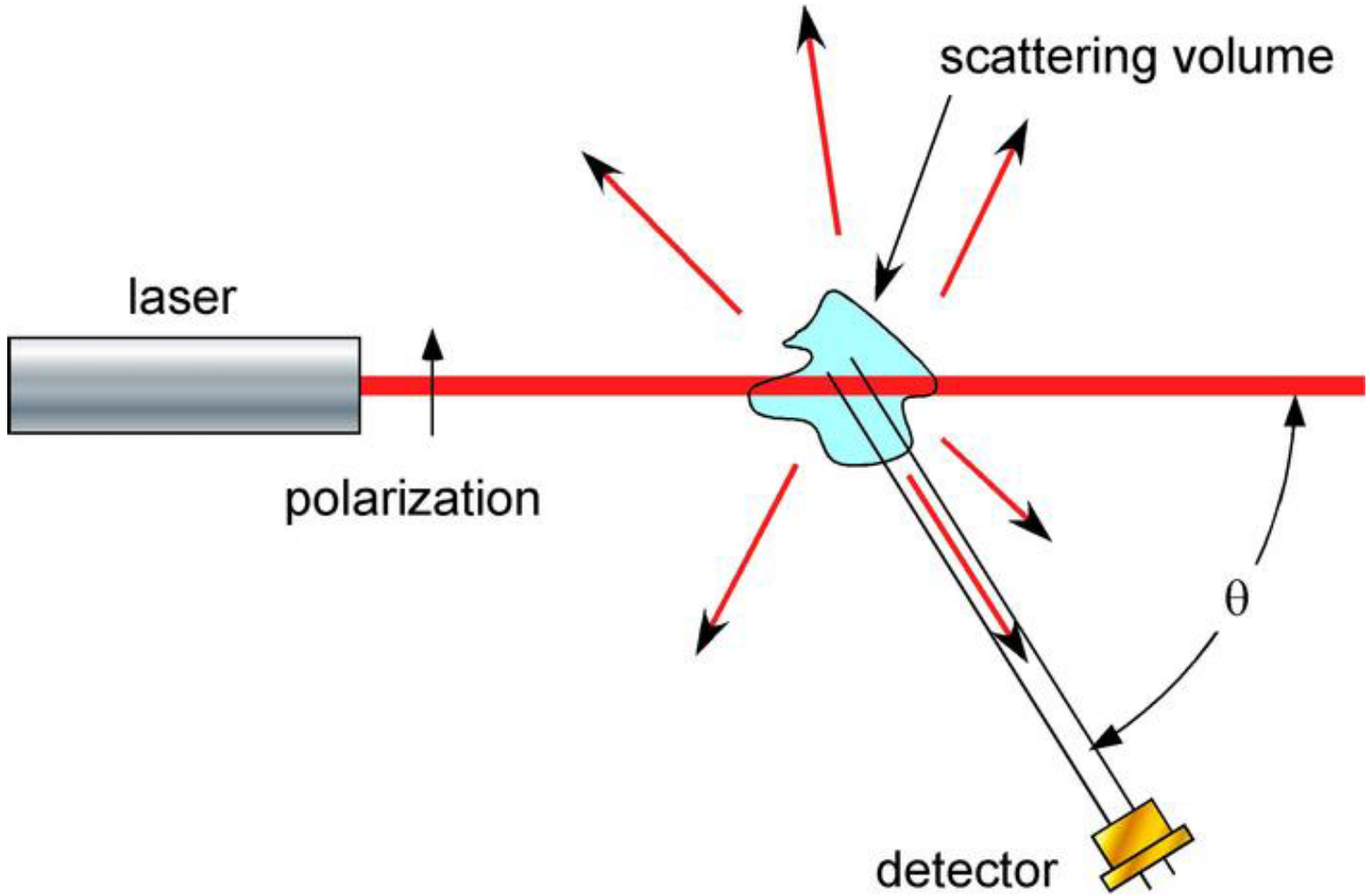
To connect the size to the molecular weight, it is necessary to make a calibration with known molecular weight standards.

Traditional SEC assumes that the sample of interest:

- has the same molecular conformation as the calibration standards
- has the same density as standards
- does not interact with the stationary phase of the column



Multi-Angle Light Scattering



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Multi-Angle Light Scattering

1st principle :

Intensity of scattered light is directly proportional to the product of the molar mass, the concentration and *dn/dc :*

$$I_s(\theta) \propto c \times M \times \left(\frac{dn}{dc}\right)^2$$

M is the average molar mass of the scattering macromolecules, which is to be determined

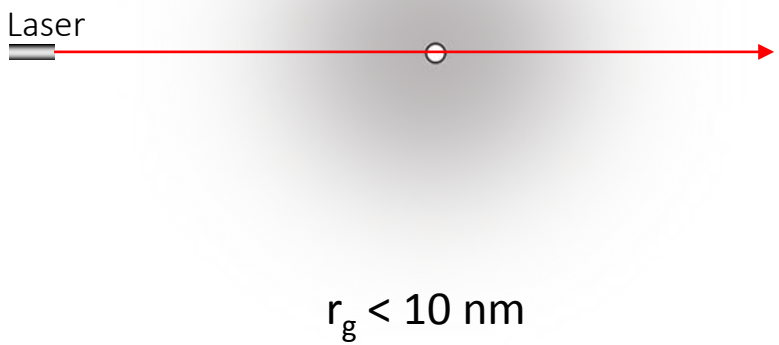
c is the concentration of the macromolecule (in mg/ml)

dn/dc is a sample specific value, which relates changes of refractive index of the solution in relation to the change of concentration.

Multi-Angle Light Scattering

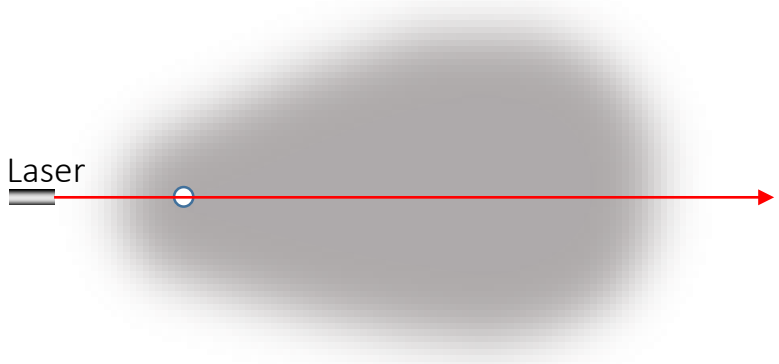
2nd principle :

The variation of scattered light with the scattering angle is proportional to the average size of the scattering molecules.
For small molecules, such as proteins, the light scattered at every angle is the same. The macromolecule scatters light isotropically.



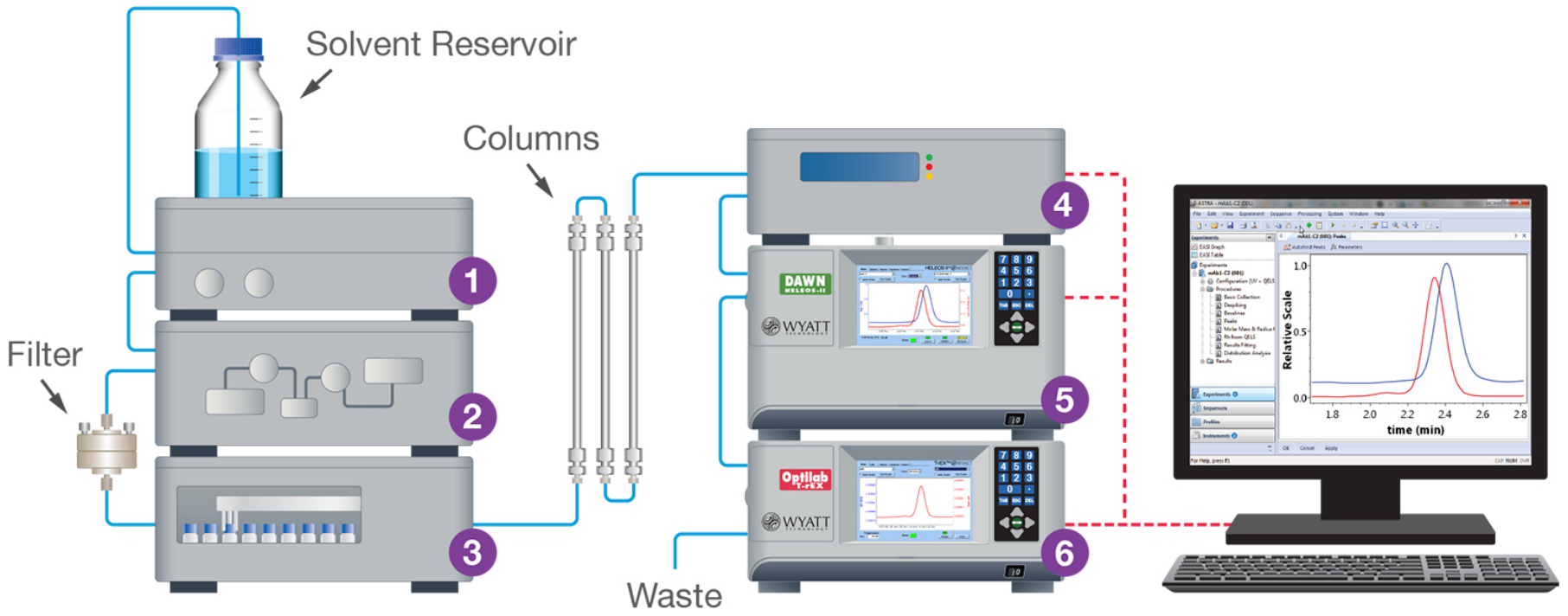
r_g < 10 nm

If an angular dependence of the scattered light is measured, then it is possible to determine the size of the molecule, *Rg*, and second virial coefficient, A_2.



r_g > 10 nm

Typical SEC-MALS configuration



1. Degasser
2. Pump
3. Autosampler or Manual Injector
4. UV Detector
5. Multi-Angle Light Scattering Detector
6. Differential Refractive Index Detector

ASTRA Software

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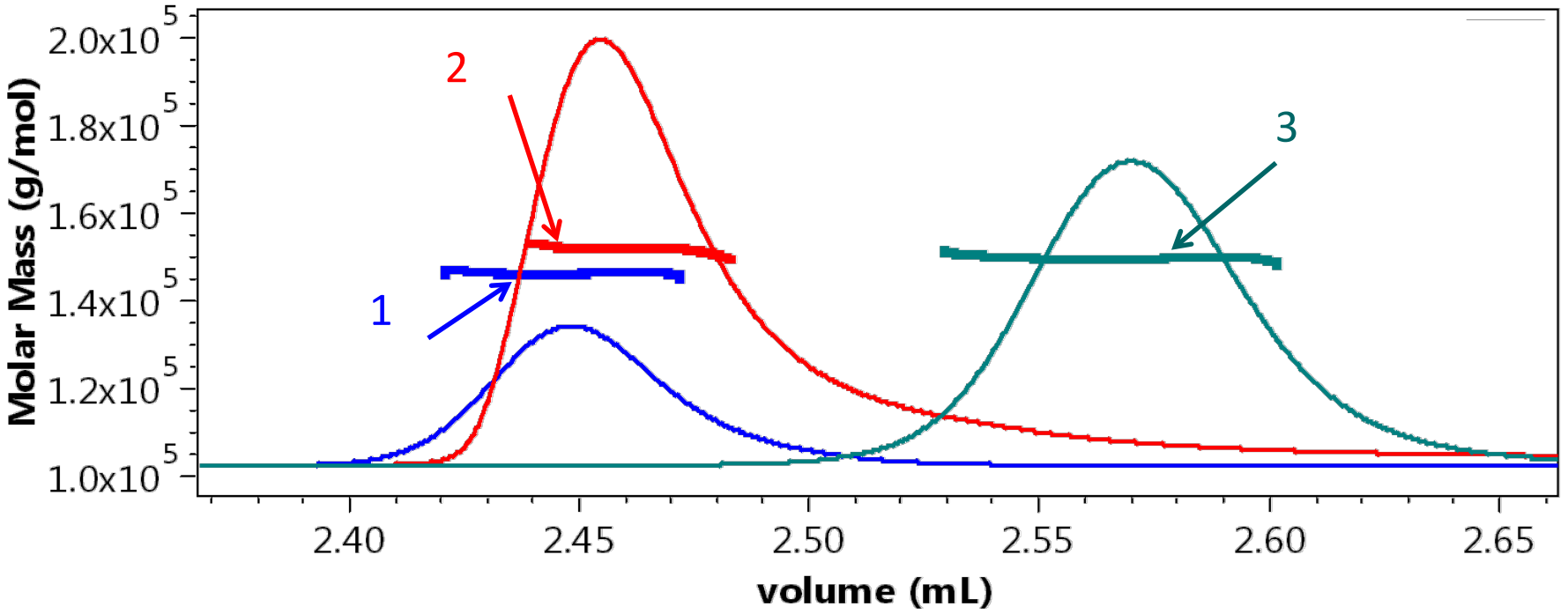
Advantage of SEC-MALS compared to SEC



Using SEC alone : according to their elution order, we would think Protein 1 has a slightly higher MW than Protein 2 and Protein 3 has a much lower MW than the other two proteins.

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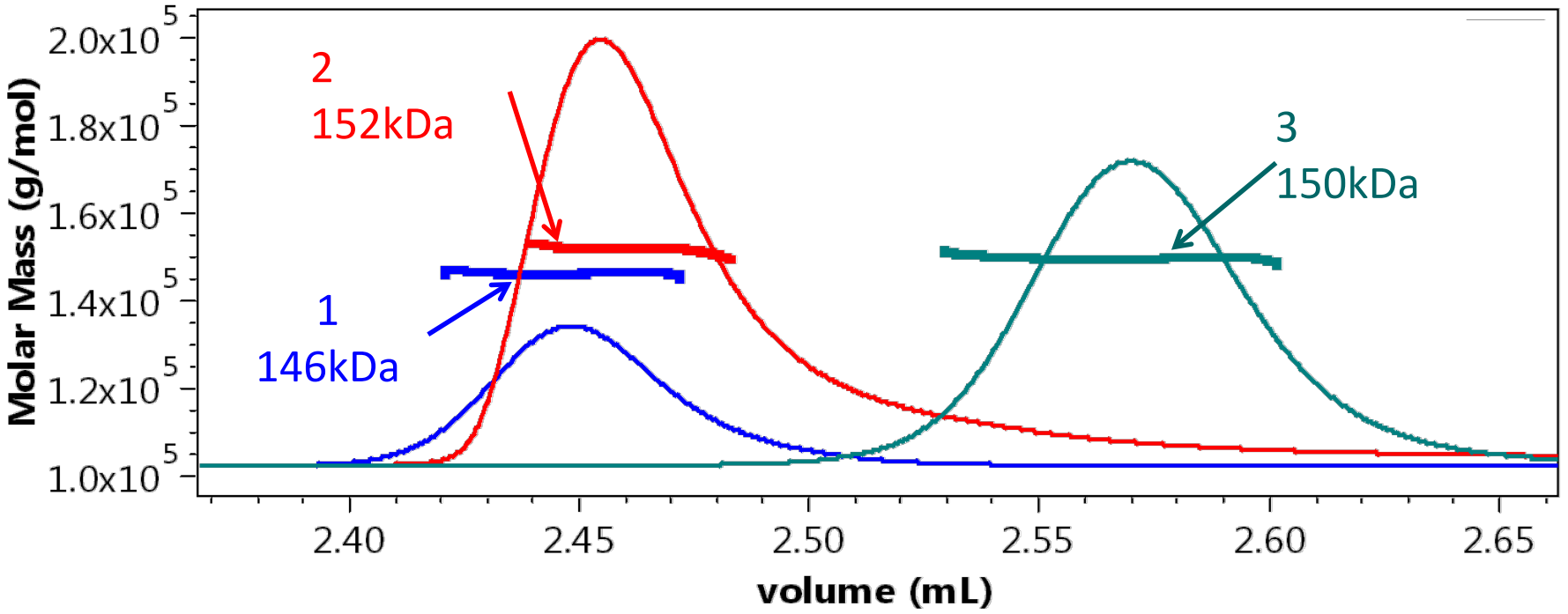
Advantage of SEC-MALS compared to SEC



Using SEC-MALS : we found Protein 1 actually has a slightly lower MW than Protein 2, 146 kDa vs. 152 kDa; and Protein 3 has a very similar MW, 150 kDa.

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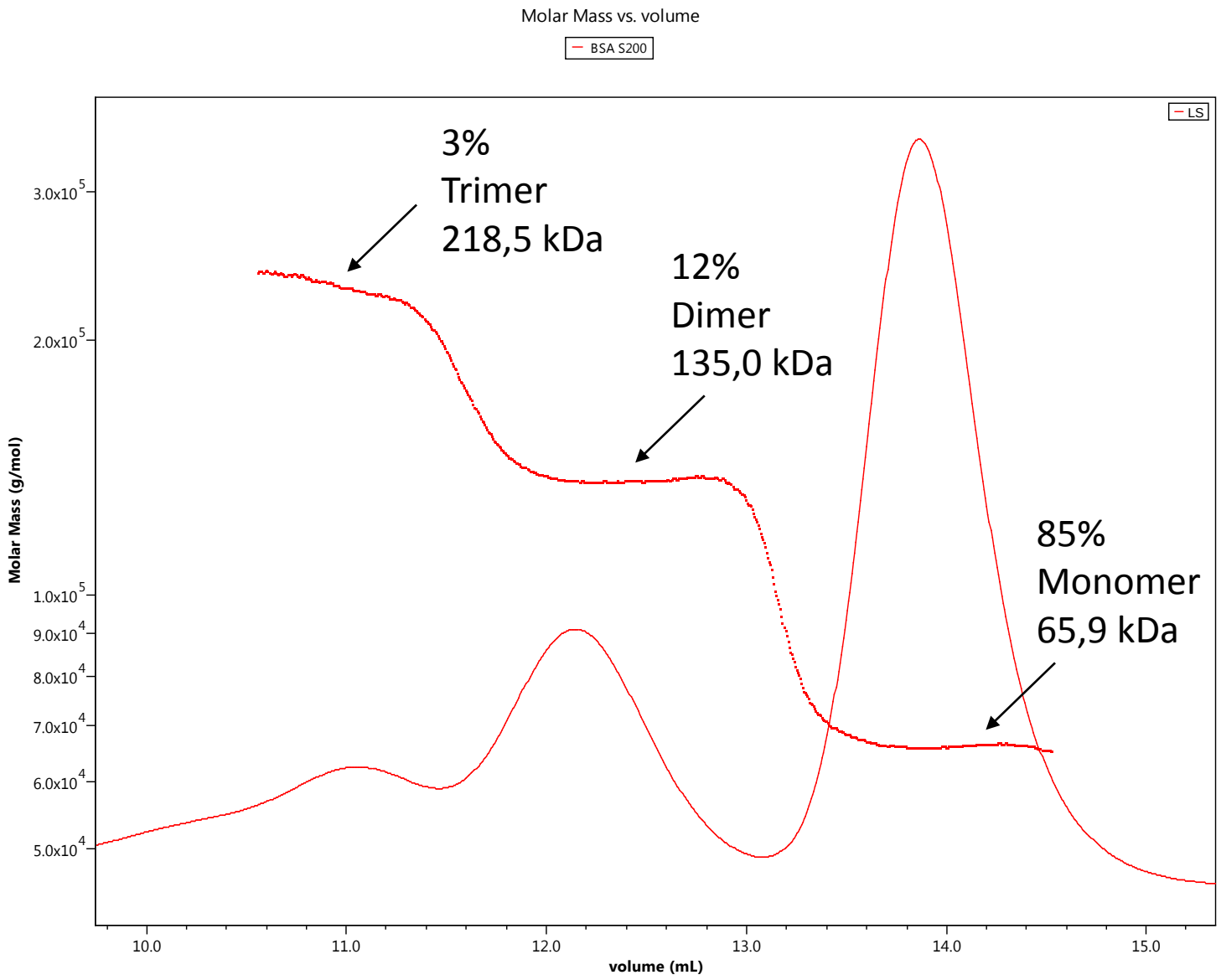
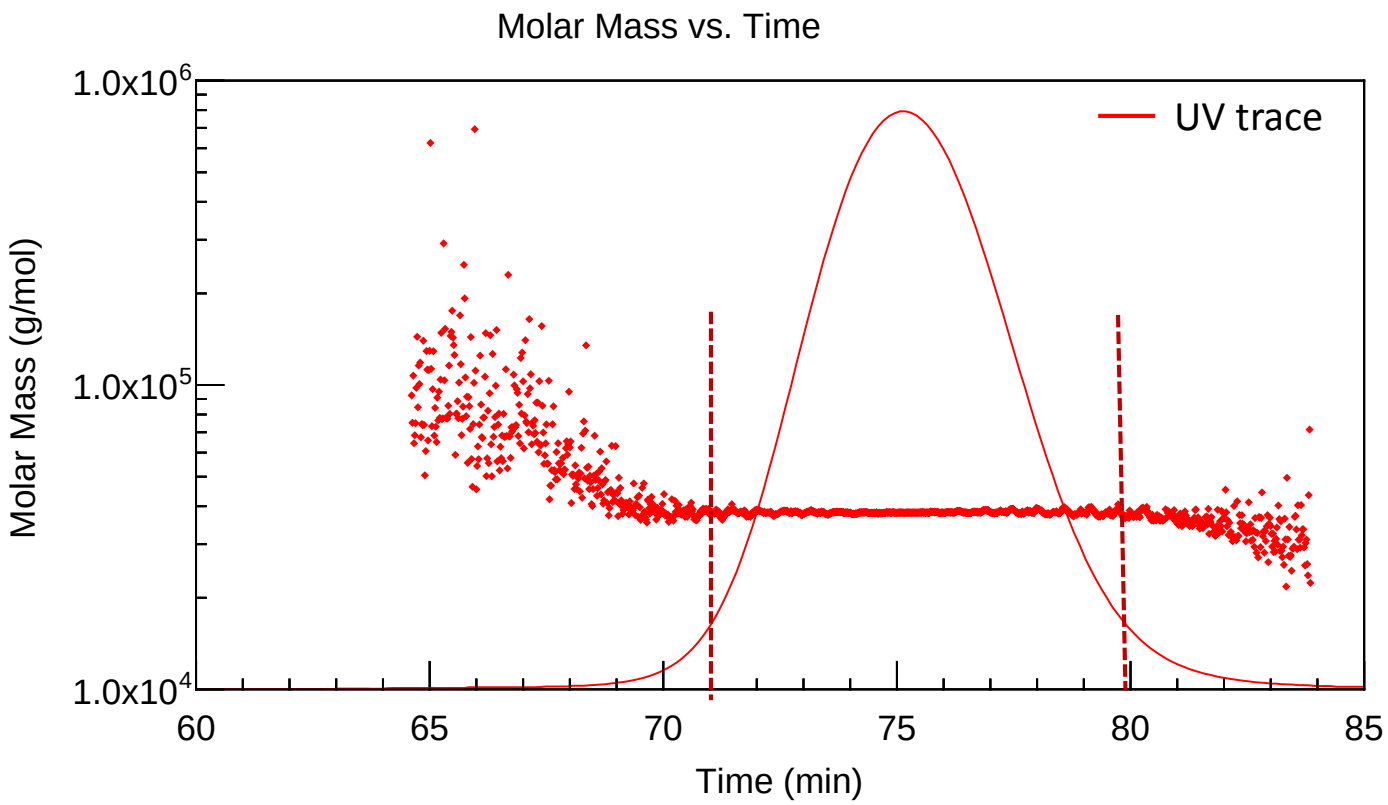
Determination of oligomeric states

Oligomeric states of the Bovine Serum Albumin (BSA)

Theoretical Mw : 66 kDa



Quality control of the purification



- Pure protein : molar mass is constant along the peak
- Molar mass corresponds to a monomer: 31 kDa

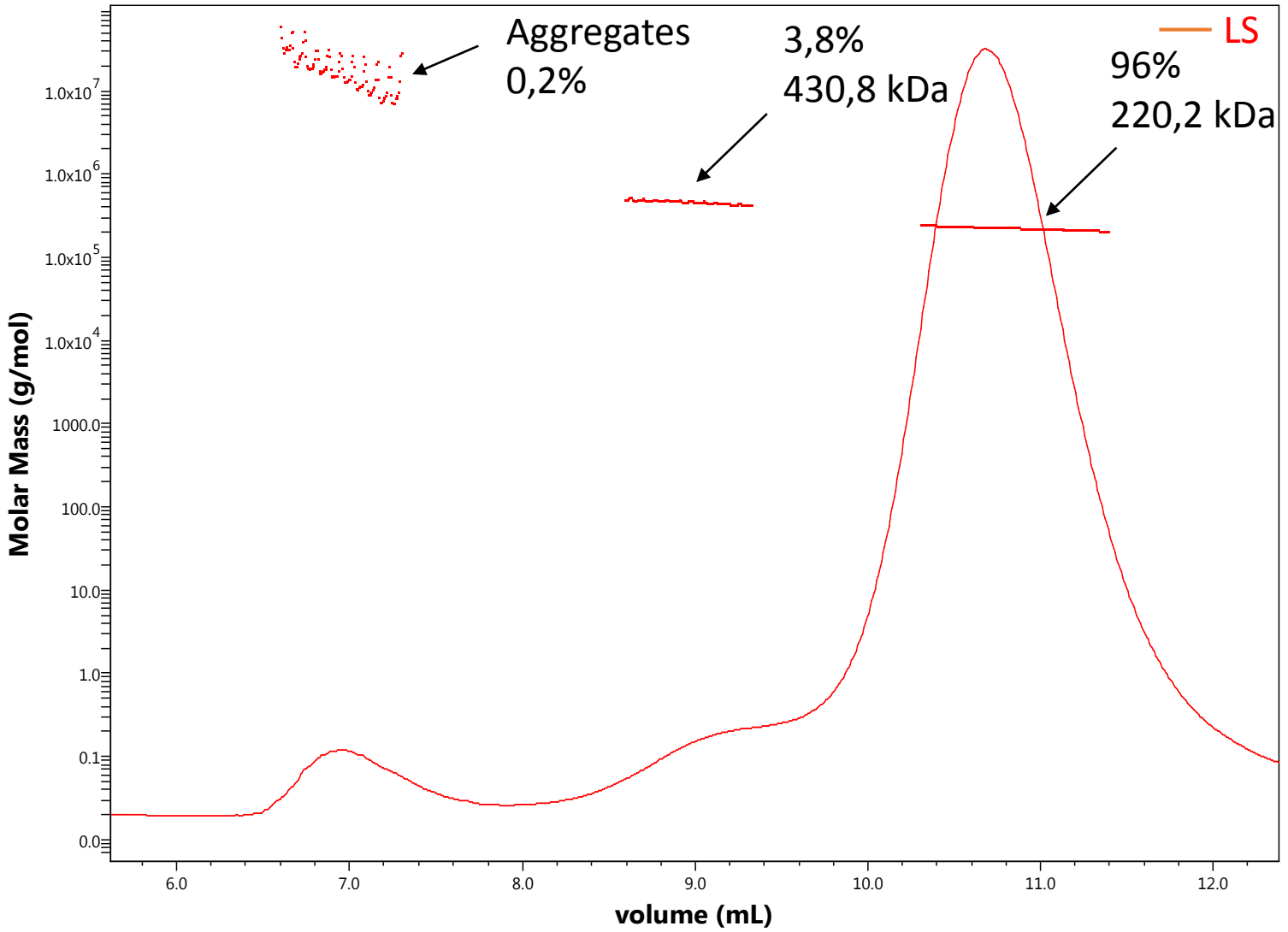
Characterisation of protein complexes

Protein complex :

- protein A = 55,3 kDa
- protein B = 105,0 kDa
- protein C = 55,4 kDa

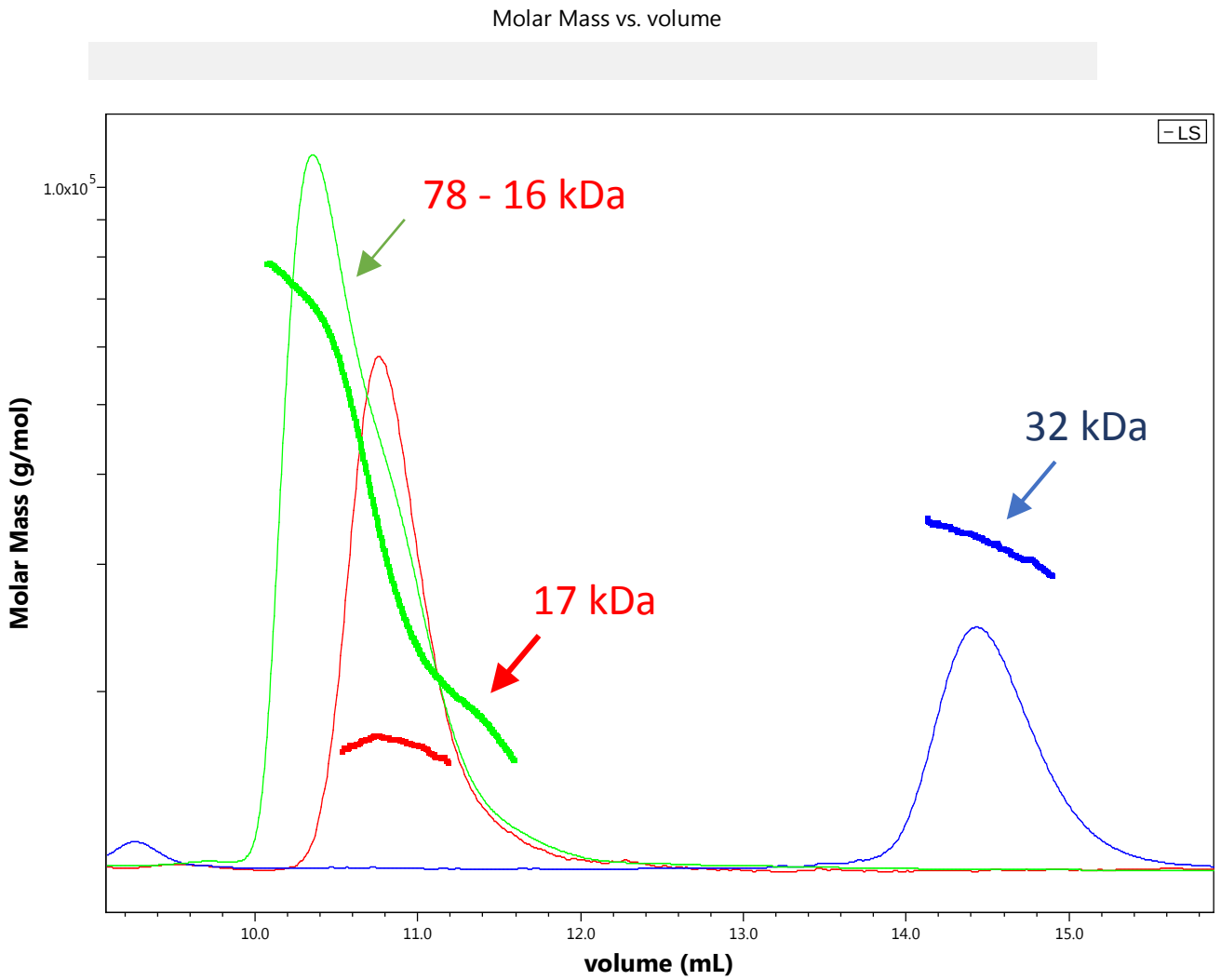
Theoretical MW
= 215,7 kDa

⟶ Homogeneous protein complex



From Nicolas Charlet (IGBMC)

Characterisation of protein complexes



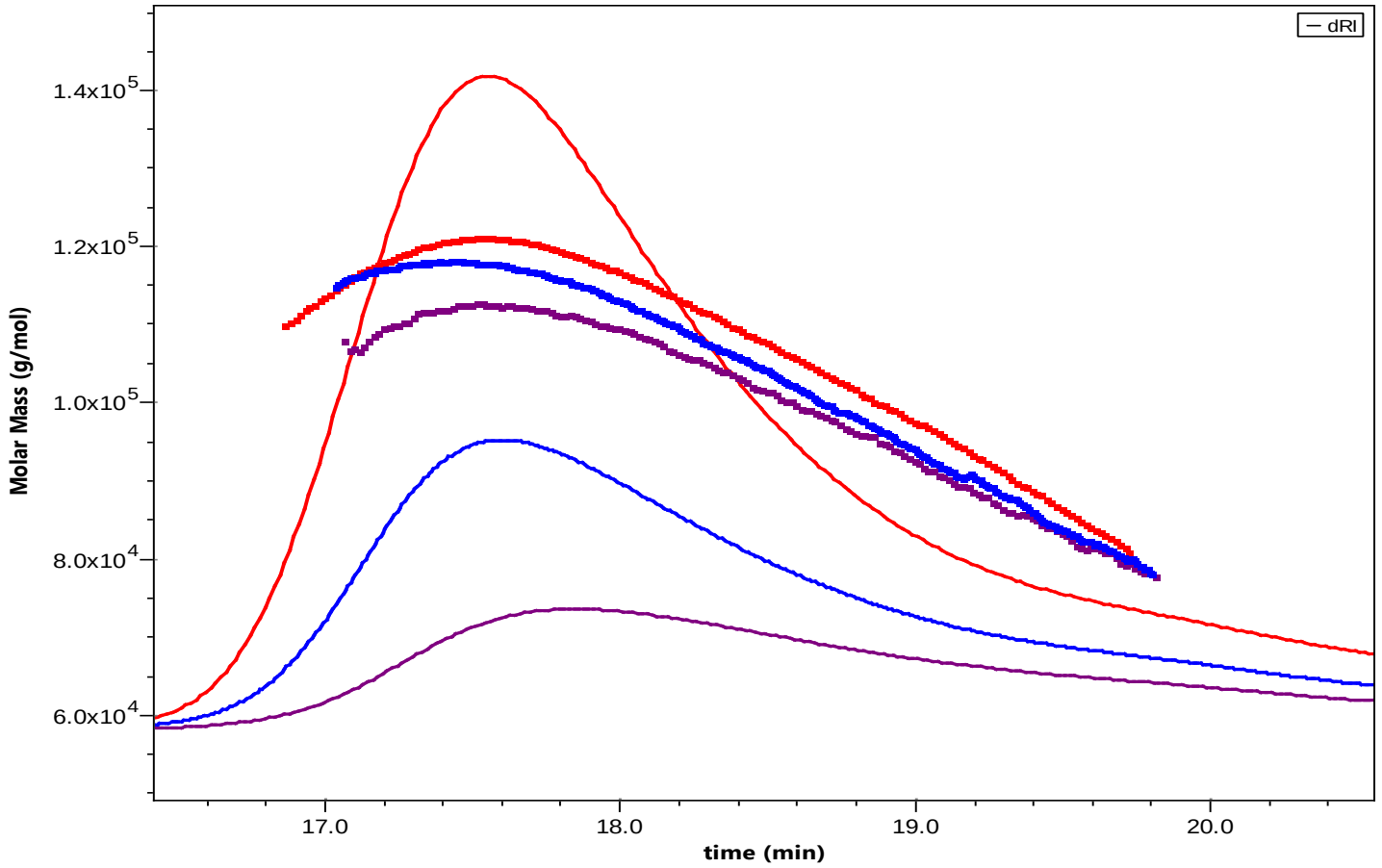
Protein 1 = 30 kDa

Protein 2= 17 kDa

The expected complex should be a dimer of Protein 1 + a monomer of Protein 2 = 77 kDa

From Vanessa Dacleu (IGBMC)

Molar mass depends on the concentration

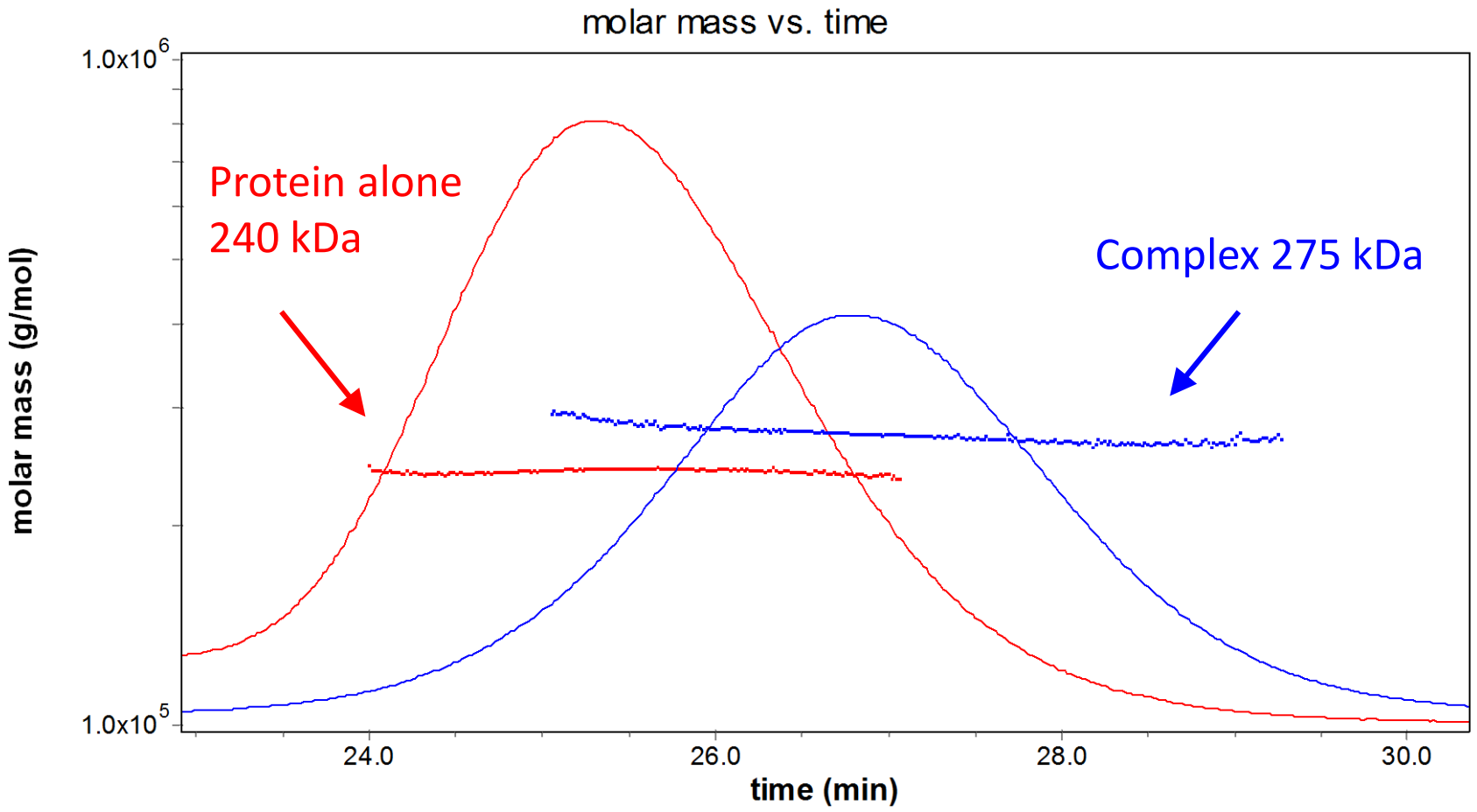


The molar mass represents equilibrium mixture of monomers and oligomers. At the top of the peak, where the concentration is highest, the equilibrium favors the oligomeric state.
Changing the injection amount leads to higher concentrations eluting from the column and, thus, higher molar mass.

WYATT TECHNOLOGY

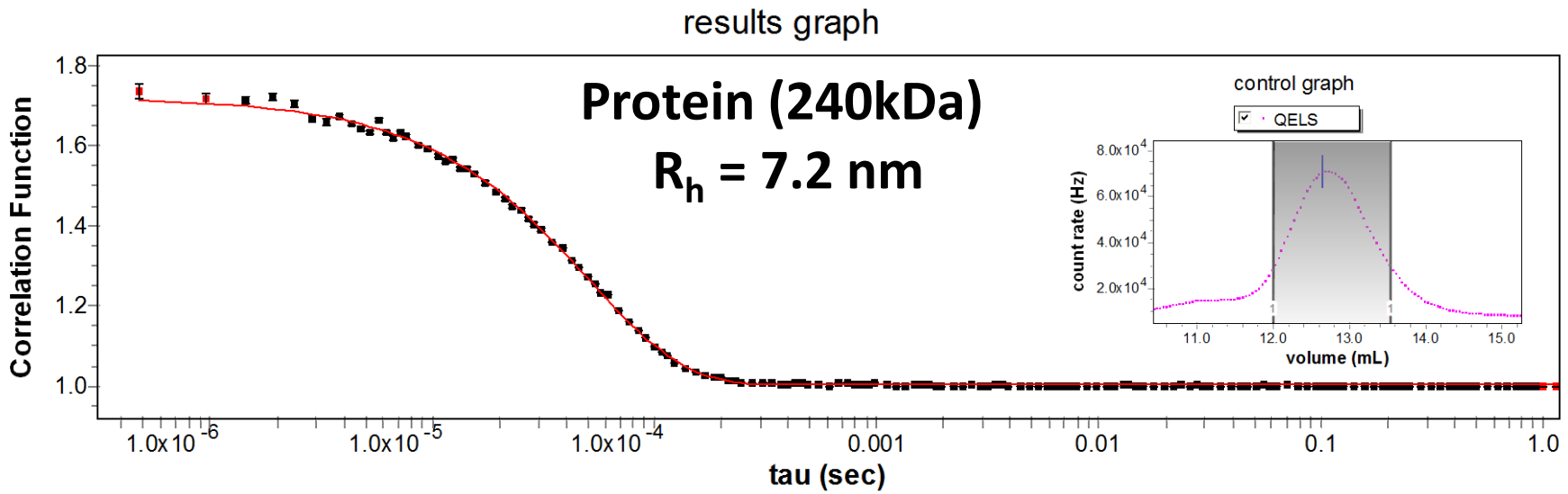
Use of DLS information

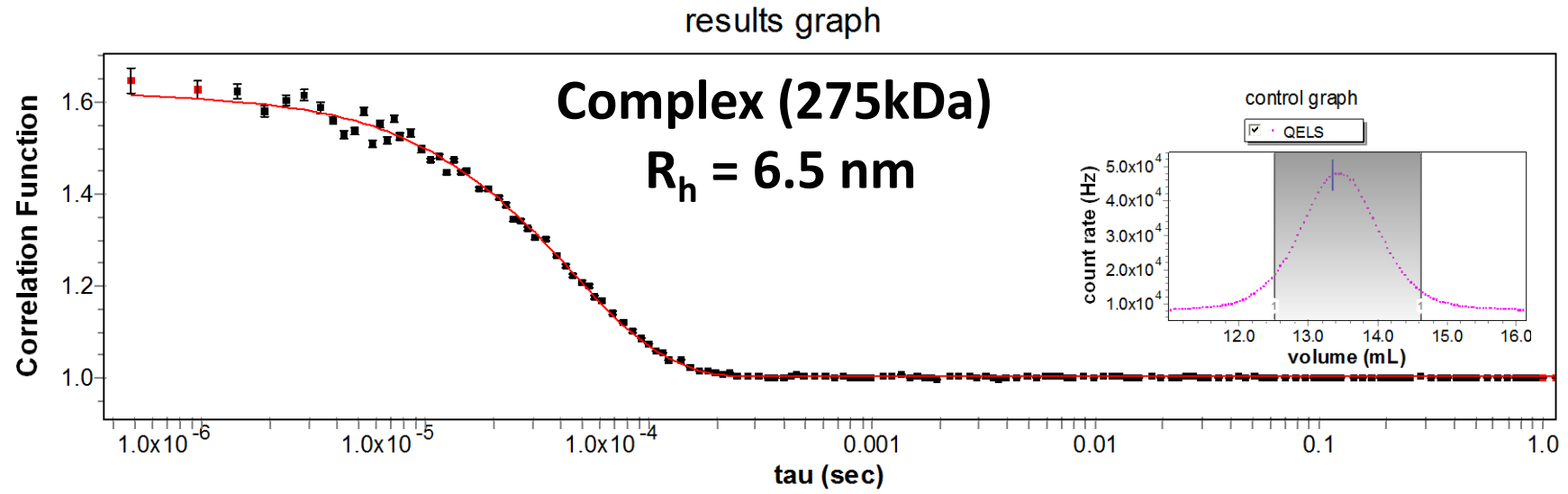
Why the complex elutes later than the protein alone?



WYATT TECHNOLOGY

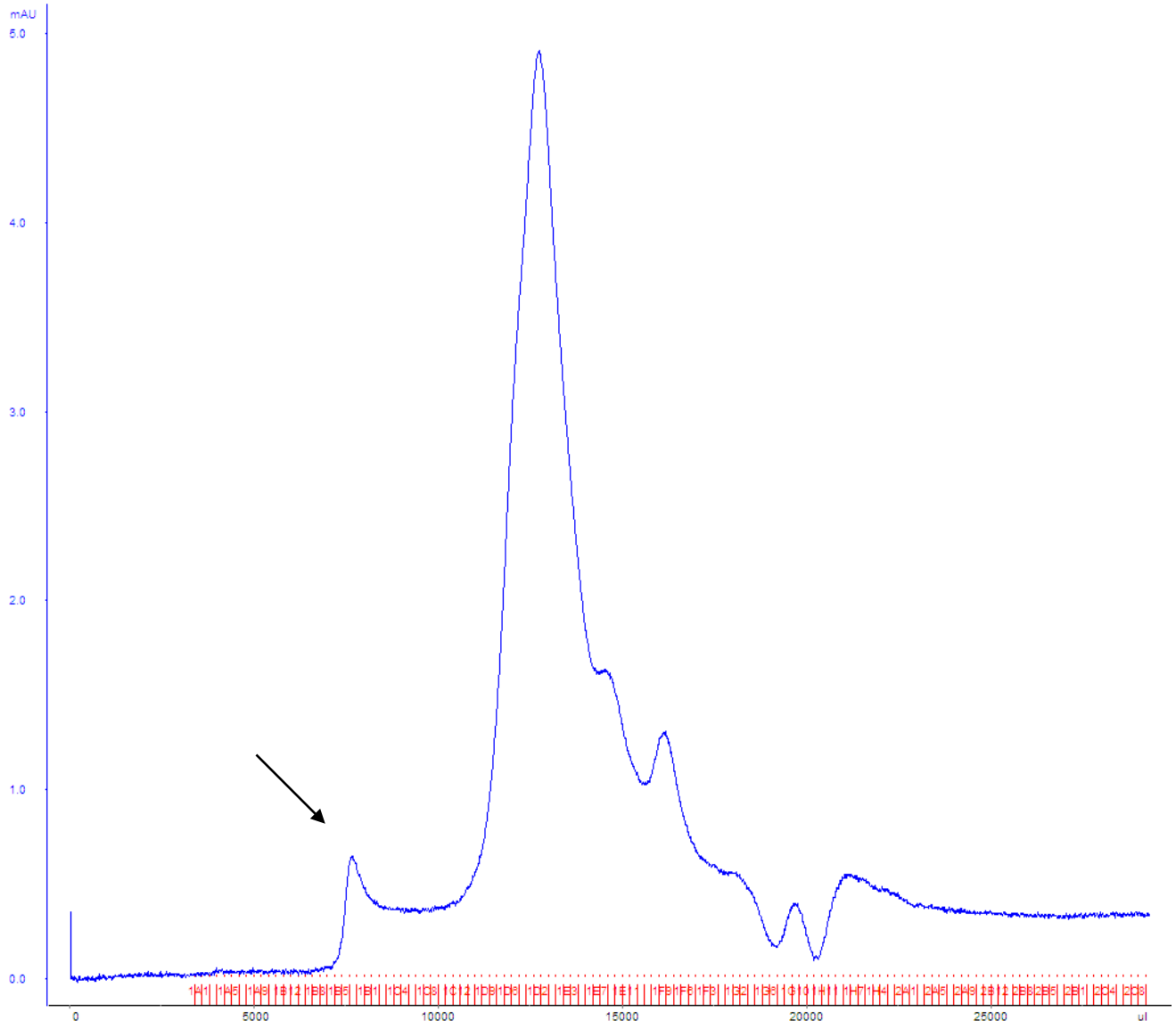
Use of DLS information





WYATT TECHNOLOGY

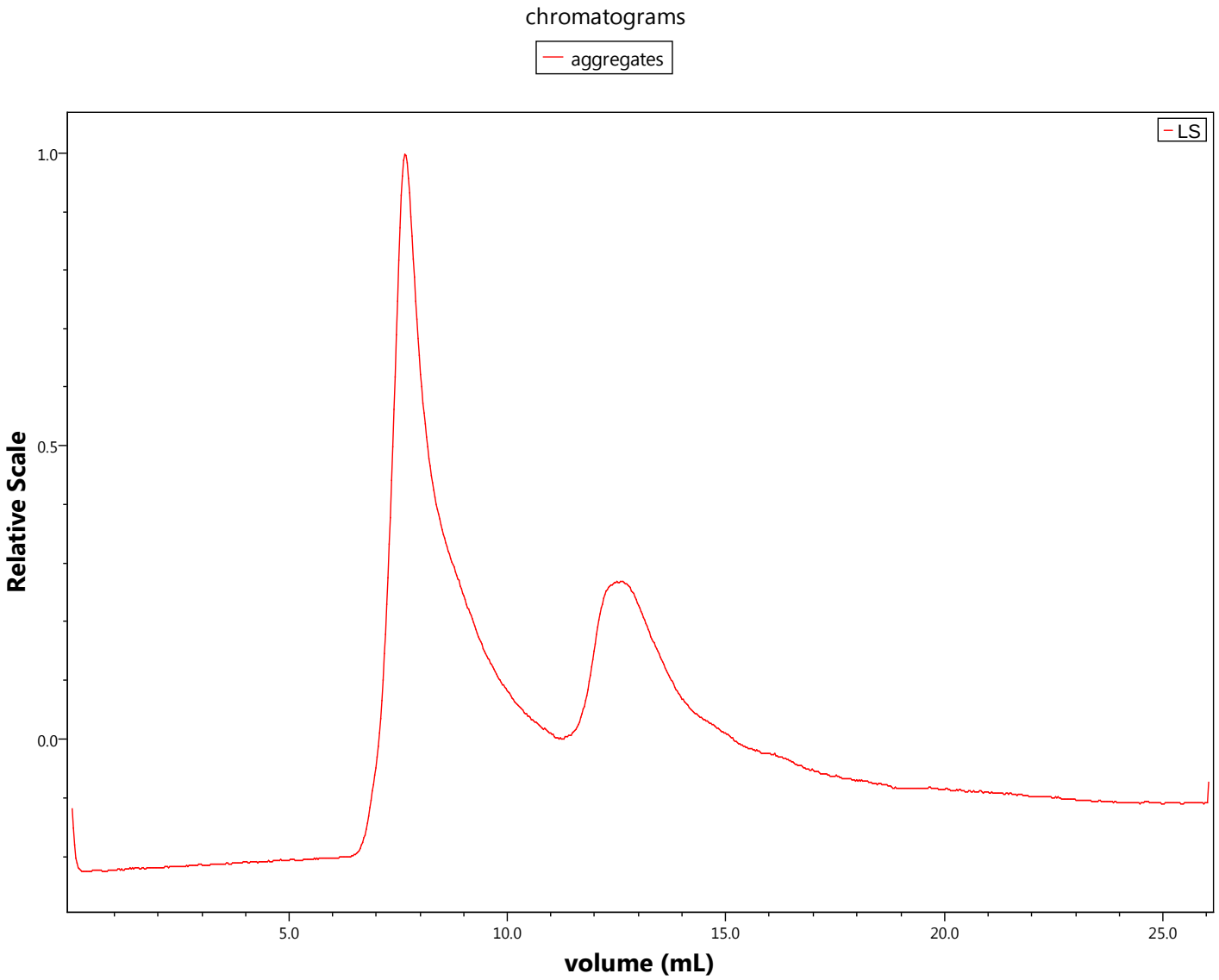
Choice of the buffer : detection of aggregates



On the chromatogram, small peak of aggregates

From Nathalie Troffer (IGBMC)

Choice of the buffer : detection of aggregates



On the LS signal, huge peak of aggregates eluting all along the SEC-MALS run.

This is not the good buffer to perform the experiment.

From Nathalie Troffer (IGBMC)

Protein conjugates analysis

Useful for characterizing a wide variety of two component systems such as:

- PEGylated and glycosylated proteins
- membrane proteins associated with detergents
- antibody-drug conjugates (ADC)

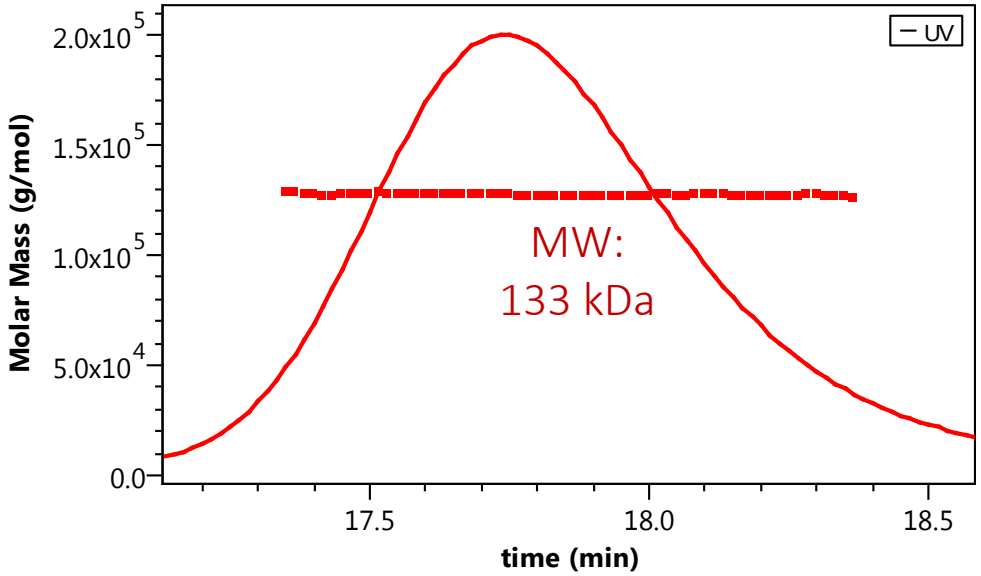
The basis of this analysis is derived from the unique combinations of three signals:

- UV absorption
- differential refractive index
- light scattering

Protein conjugates analysis

Characterisation of a glycosylated protein

Theoretical Mw of the monomer : 113 kDa

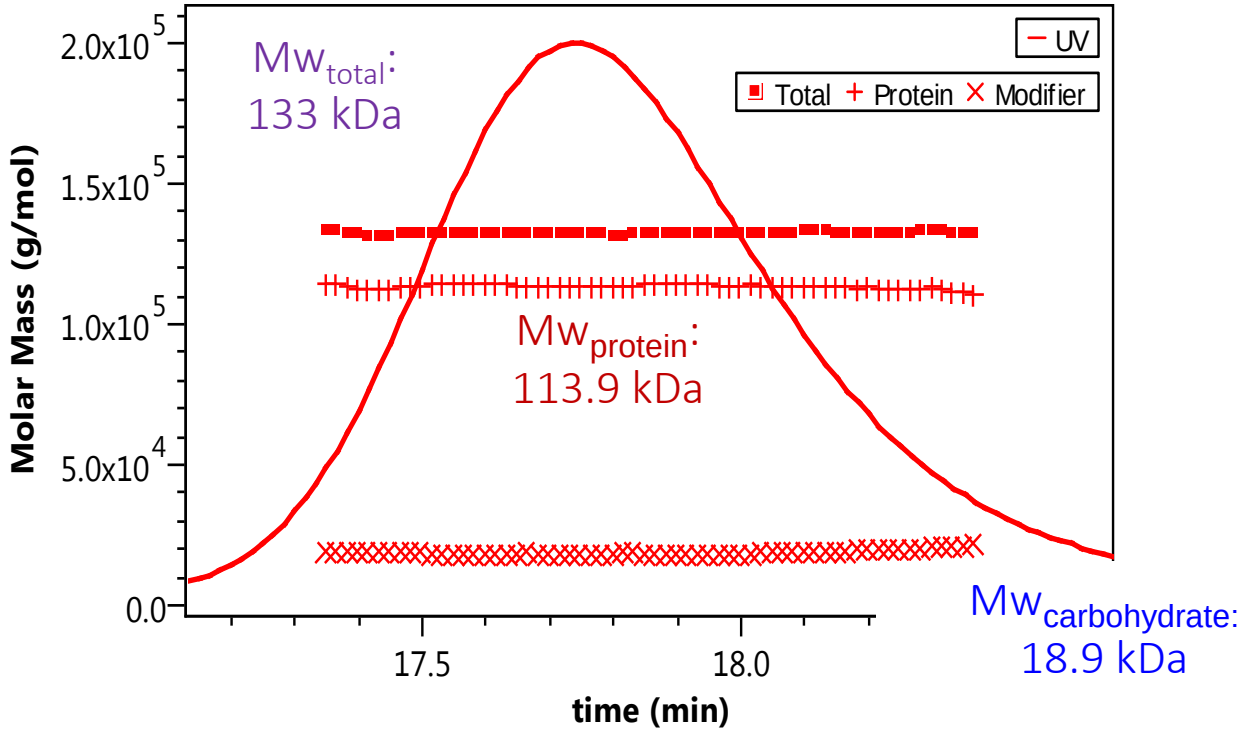


Problem : the experimental molar mass doesn't correpond to a monomer, neither a dimer.

WYATT TECHNOLOGY

Protein conjugates analysis

Characterisation of a glycosylated protein

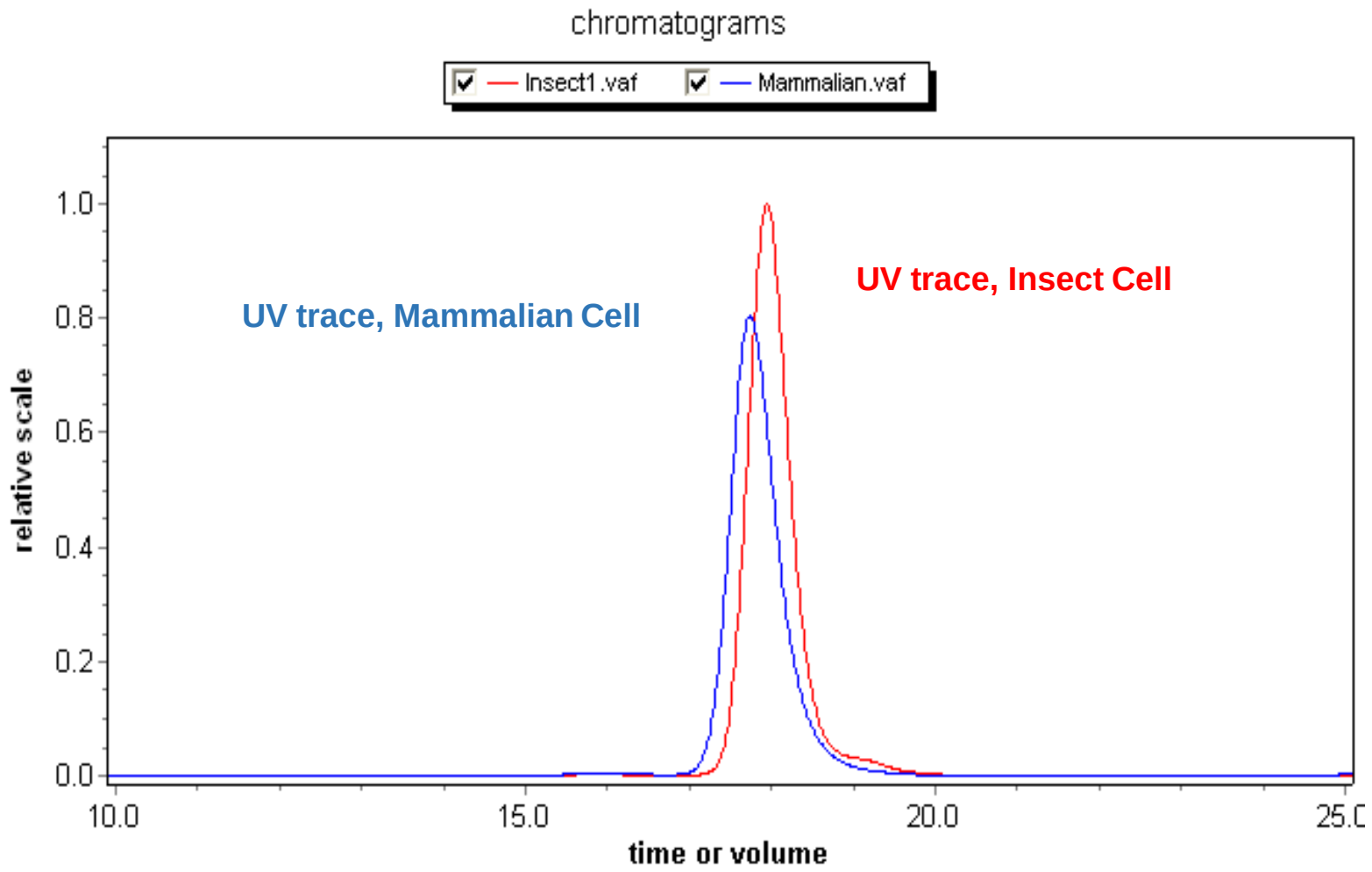


Conclusion: the protein is in a monomeric form, with glycosylation.

WYATT TECHNOLOGY

Protein conjugates analysis

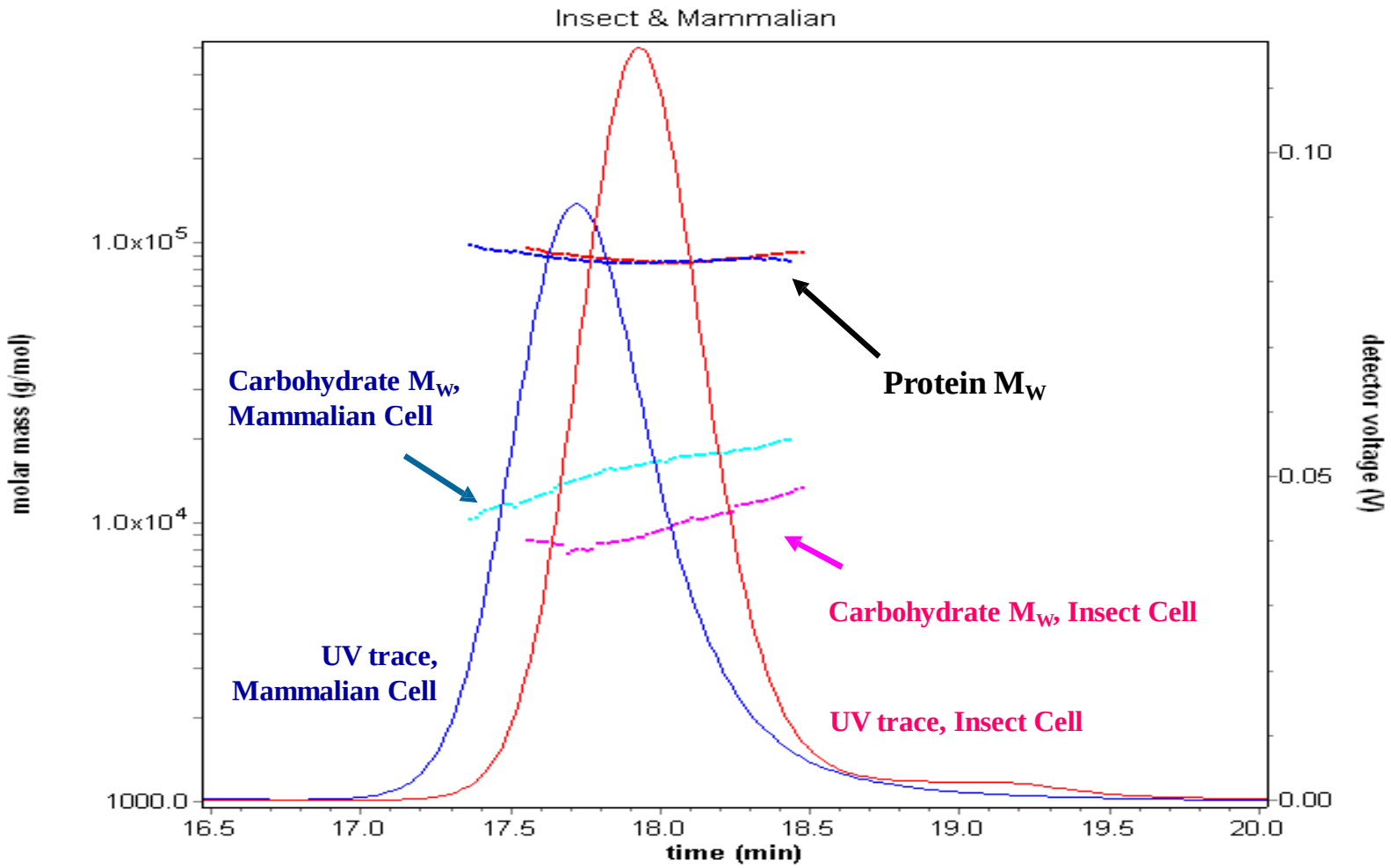
Protein expressed from mammalian or insect cells



WYATT TECHNOLOGY

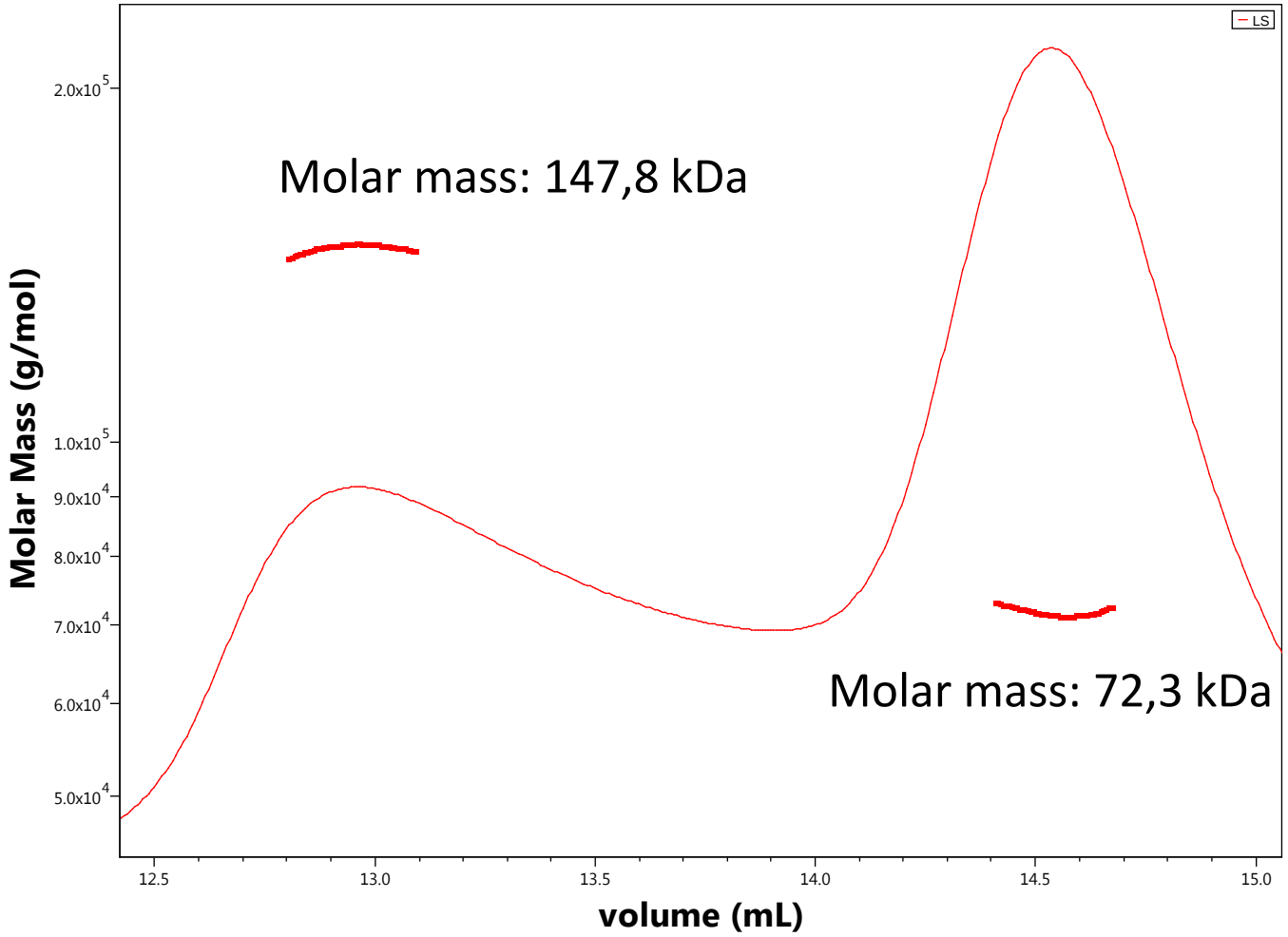
Protein conjugates analysis

Protein expressed from mammalian or insect cells



Protein conjugates analysis

Characterisation of a protein-detergent complex



Theoretical Mw of the protein : 63 kDa

Two populations:

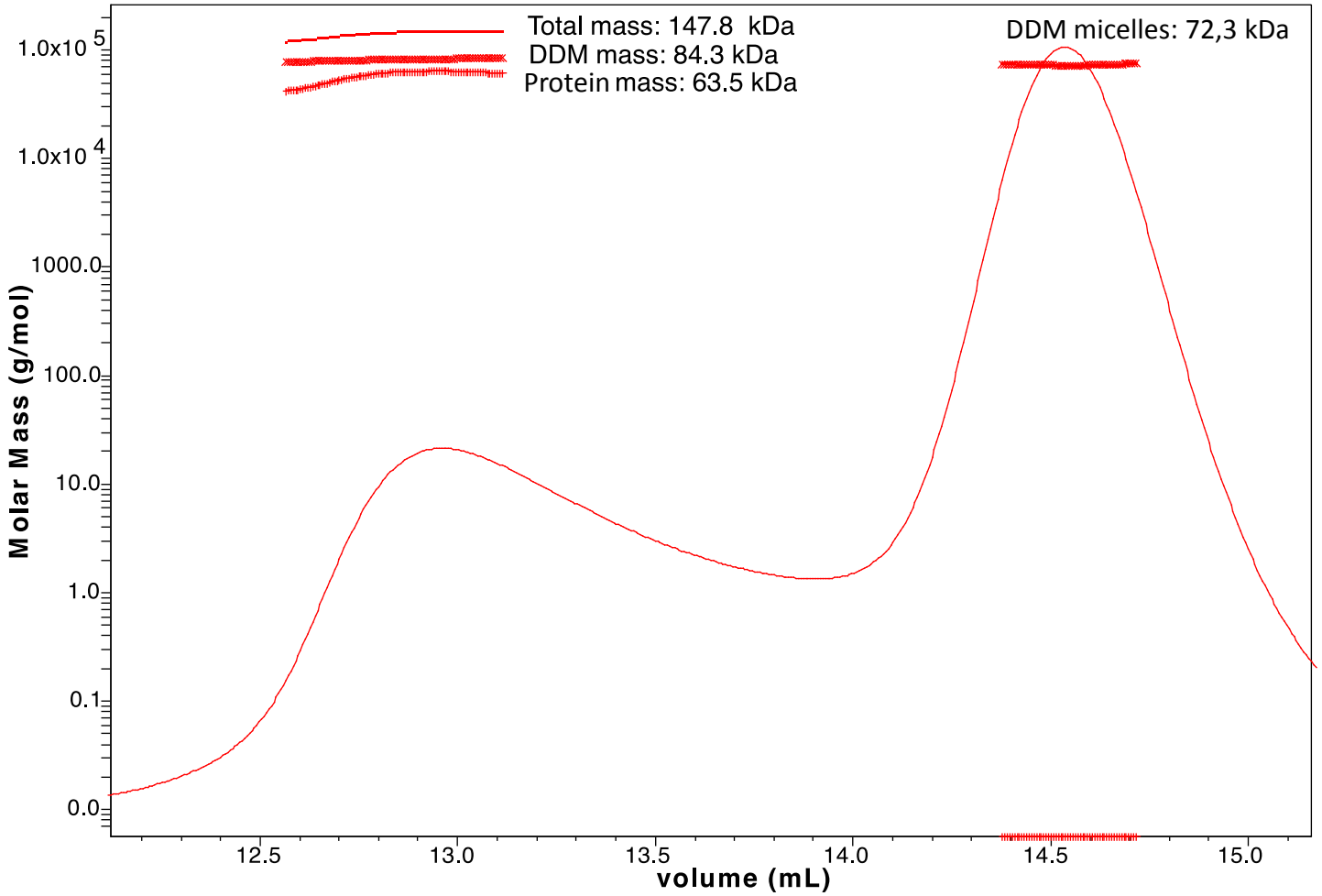
Peak 1 : 147,8 kDa

Peak 2: 72,3 kDa

From Béatrice Roche (INSTRUCT access)

Protein conjugates analysis

Characterisation of a protein-detergent complex



For the protein:
(dn/dc) = 0.185
UV extinction coefficient at 280 nm
= 0.912 ml mg^{-1} cm^{-1}

For the detergent:
(dn/dc) = 0.1435
UV extinction coefficients at 280 nm
= 0.044 ml mg^{-1} cm^{-1}

From Béatrice Roche (INSTRUCT access)

Conclusions

- MALS coupled to SEC provides absolute molar mass information at every point of the eluting sample
- This allows identification of the protein and its association state and to detect traces of higher order aggregates
- MALS combined with UV and RI detection is a powerful tool to characterize protein conjugates

Advantages of SEC-MALS

- In solution
- Buffer versatility
- Low protein requirement
- No modification of the proteins
- Quantitative
- Quick data acquisition & analysis

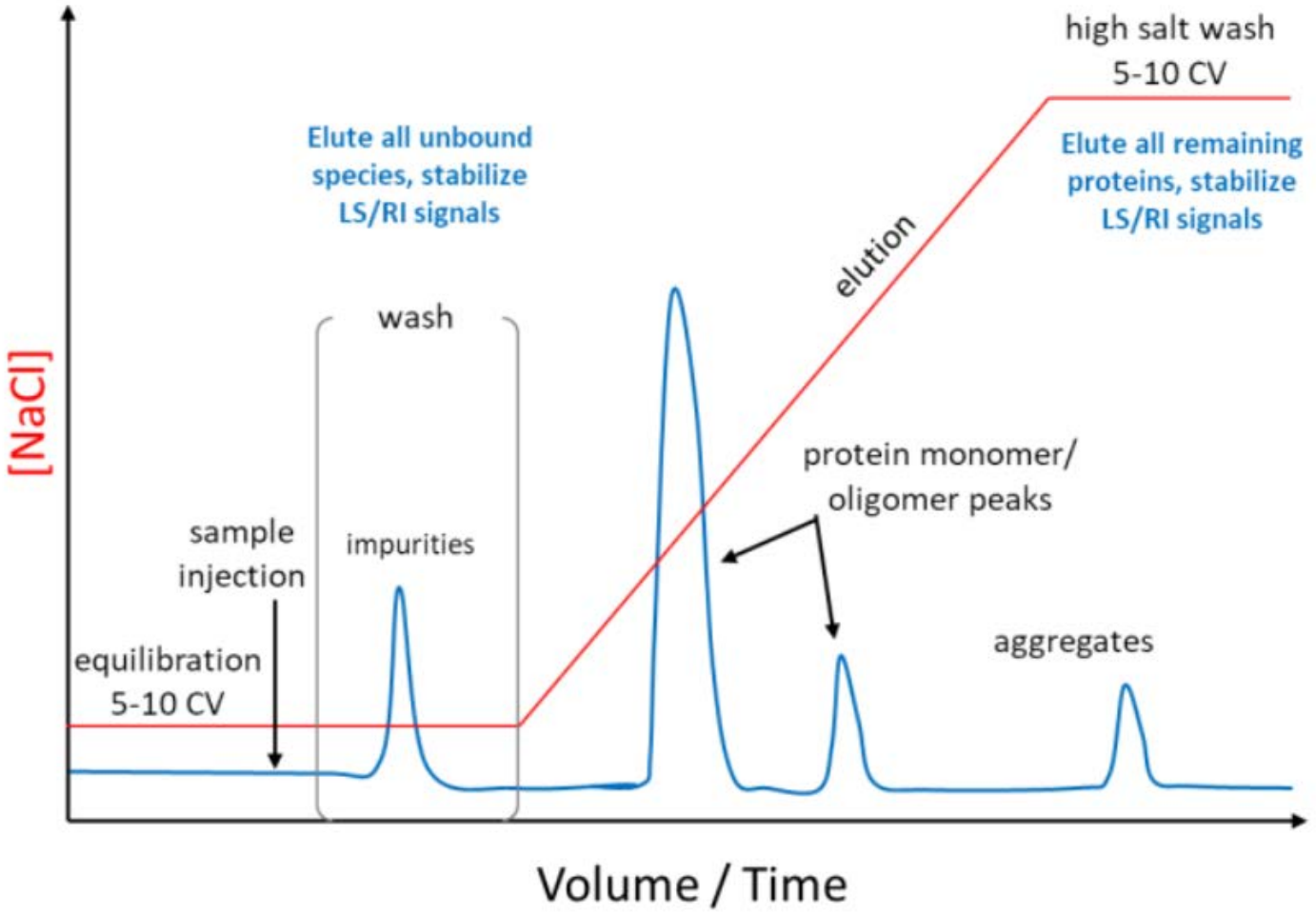
Limitations of SEC-MALS

- Molecules with the same size cannot be separated and properly analyzed
- SEC-MALS should be used only for well-resolved peaks
- Aggregates have a very intense light scattering signal, and thus the presence of even low amounts of aggregates in the protein peak can introduce a significant error into the calculated molar mass
- SEC-MALS usually requires extensive equilibration for achieving a clean baseline signal

Alternative technique : IEX-MALS

IEX separates macromolecules according to their surface charge, based on different ionic interactions with the support matrix.

More parameters can be optimized compared to SEC (gradient slope, salt composition and concentration, buffer pH, matrix...)

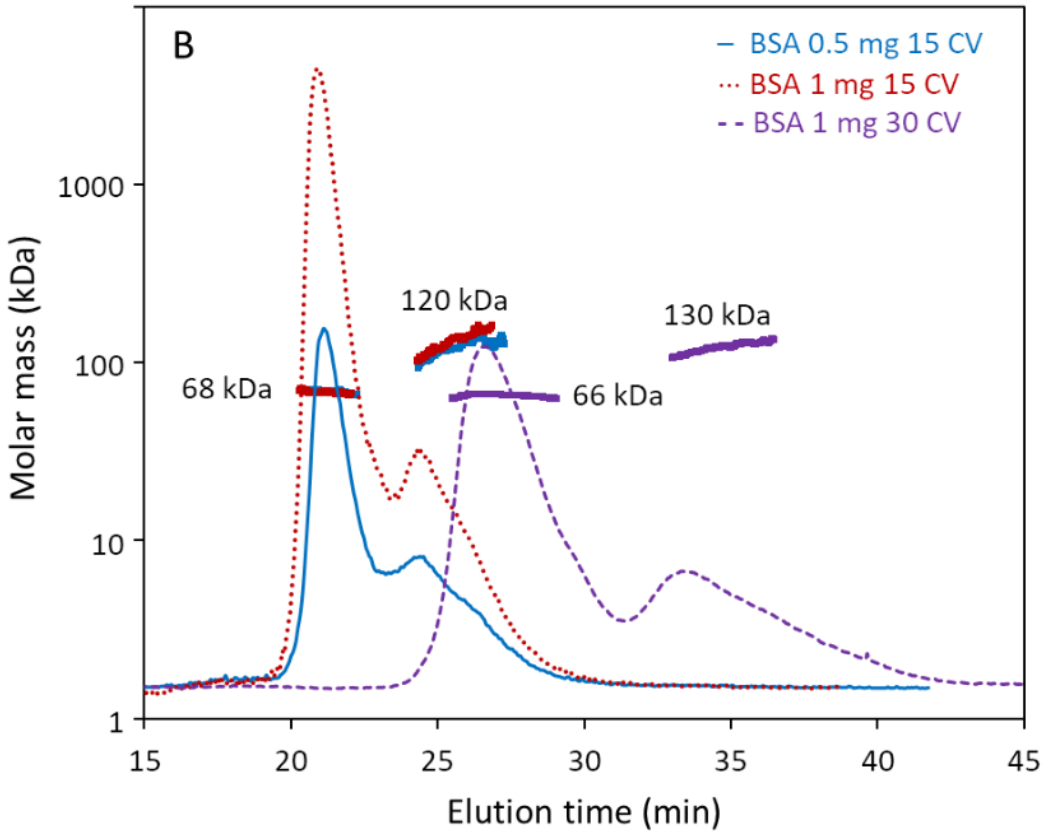


Amartely, H *et al.* (2018)

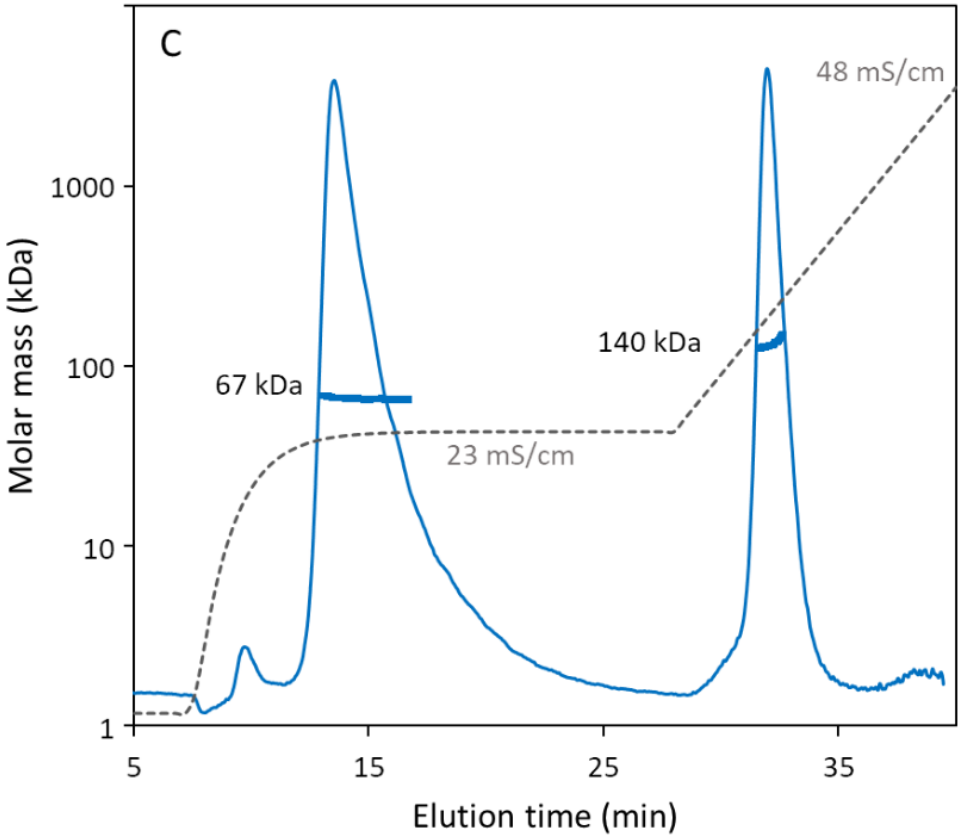
Alternative technique : IEX-MALS

AIEX-MALS of BSA with different gradient programs:

15-70% of 500 mM NaCl over 15CV and 30CV



35% step and 35-100% gradient over 10CV



Alternative technique : IEX-MALS

- The different separation technique used in IEX-MALS provides an additional way to absolute protein characterization, making it complementary to SEC-MALS.

- This technique should be employed to characterize samples that cannot be separated by standard SEC because the species present have similar hydro-dynamic size or otherwise co-elute.

Aknowledgments

The Platform team members:

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Florence GRANGER

Alastair McEWEN

Marie-Christine POTERSZMAN