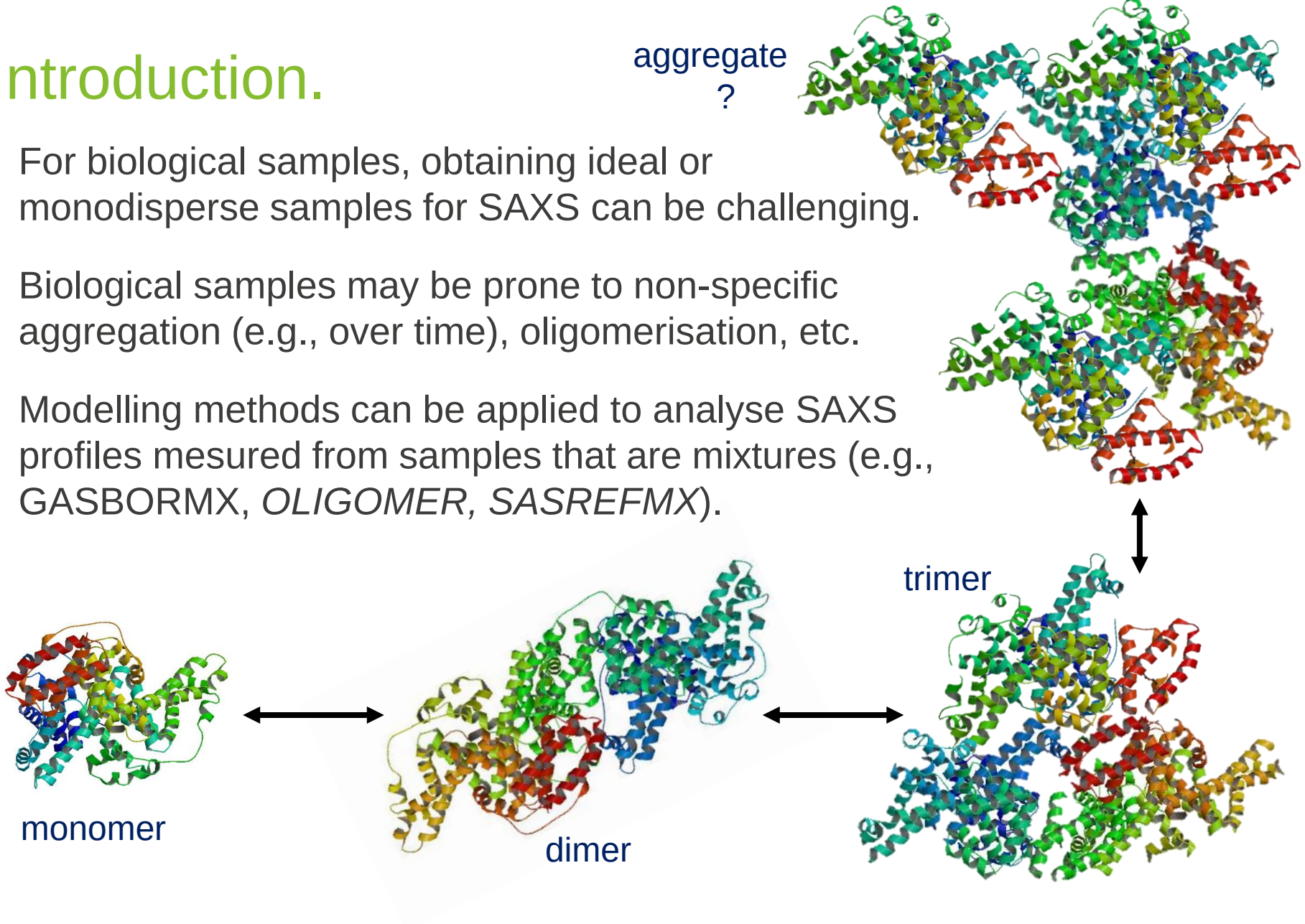
In Line Purification of Equilibrium Mixtures.

Cy Jeffries
EMBL Hamburg

EMBL
40 YEARS | 1974–2014

Introduction.

- For biological samples, obtaining ideal or monodisperse samples for SAXS can be challenging.
- Biological samples may be prone to non-specific aggregation (e.g., over time), oligomerisation, etc.
- Modelling methods can be applied to analyse SAXS profiles mesured from samples that are mixtures (e.g., GASBORMX, *OLIGOMER, SASREFMX*).



The general scattering relationship.

For a mixture $$I(q) = \sum_i^n [(\Delta\rho_i V_i)^2 P_i(q)] S(q)$$

Component 1 scattering…plus

$$I_1(q) = N_1(\Delta\rho_1 V_1)^2 P_1(q)$$

…component scattering contributions

Component 2 scattering…plus

$$I_2(q) = N_2(\Delta\rho_2 V_2)^2 P_2(q)$$

Component 3 scattering…etc

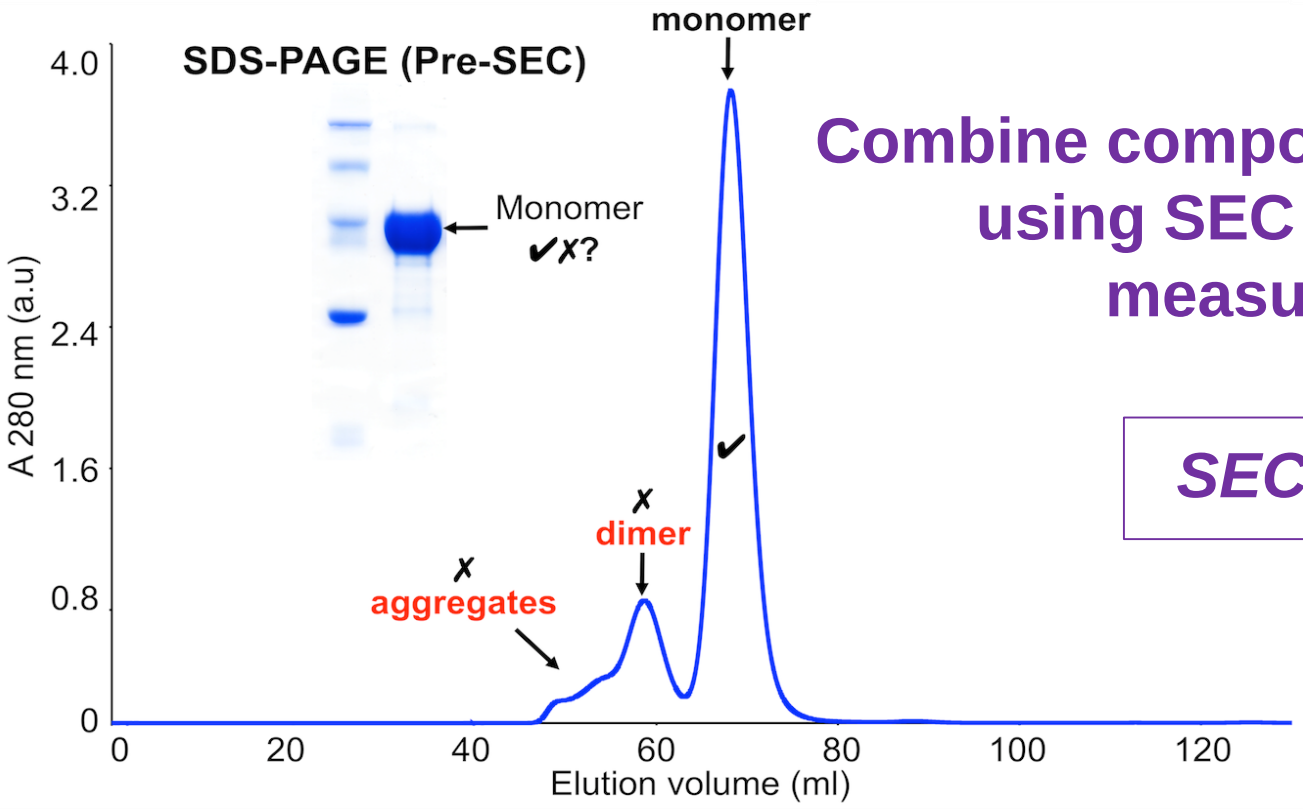
$$I_3(q) = N_3(\Delta\rho_3 V_3)^2 P_3(q)$$

- For SAXS, quantifying the individual component scattering contributions from a non-ideal (mixed system) is sometimes not so easy.

- **The answer?** Physically isolate the individual components and measure the SAXS data from each separated component.

Physical separation.

- Size exclusion chromatography can be used to separate the components of mixtures.



Combine component separation using SEC with the SAS measurements.

SEC-SAS

Jeffries et al. (2015) *Under review*. Nat. Protocols

Background to SEC

- SEC is extremely useful for separating components of already-pure equilibrium systems (e.g., monomer–oligomers) or removing trace non-specific aggregates from a sample.

Preparative SEC – you use in the laboratory for purifying proteins, macromolecules, etc

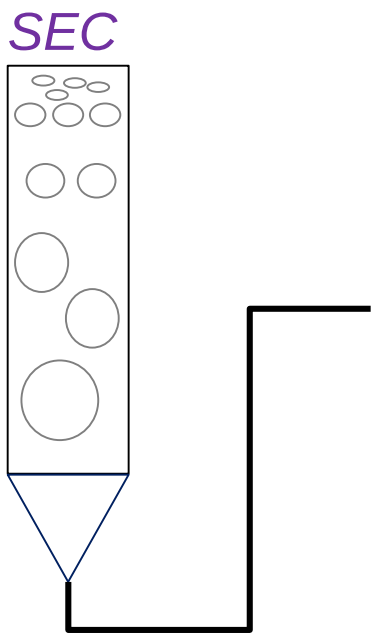
Analytical SEC – you use for the analysis of proteins and macromolecules that are already essentially very pure.

SEC-SAS is an analytical technique.

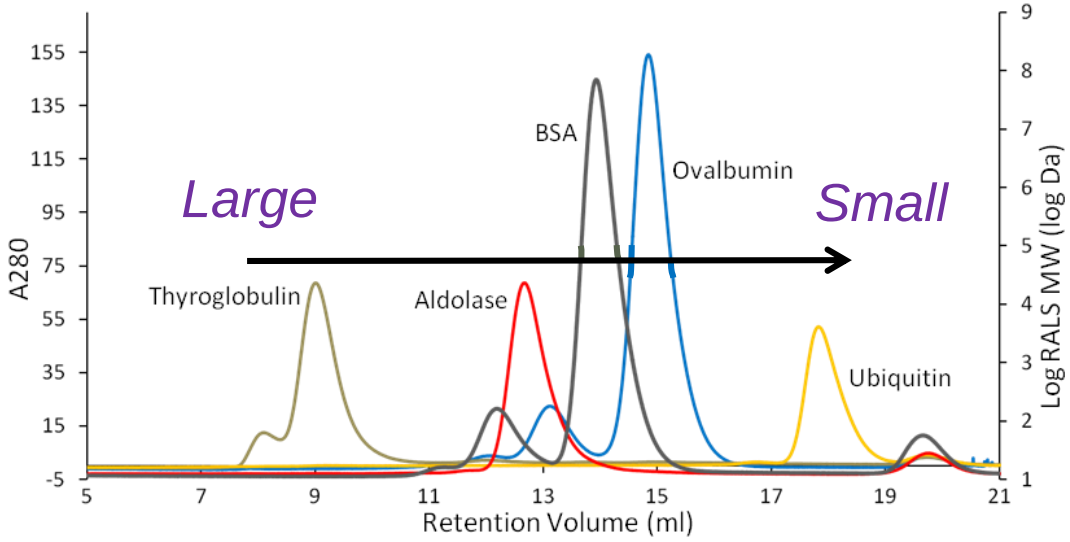
SEC with UV.

- Size exclusion chromatography, *is supposed to* separate particles based on size, typically monitored using UV (UV-SEC).

- Larger particles are *supposed to* elute first from a SEC column, followed by smaller and smaller particles.



- *log-MW separation of macromolecules*



Is a standardised SEC column sufficient to determine the MW of eluting components?

- No.
- A standardised SEC column, *in principle*, can be used to estimate the molecular weight of a particle…in an ideal world…but in reality…
- What SEC does is separate macromolecules based on the hydrodynamic behavior of macromolecules in solution flowing through a *column matrix*.
- Therefore, the presence of the matrix can have unforeseen (and typically non-ideal) consequences on the hydrodynamic behaviour of macromolecules that are otherwise impossible to predict *a-priori*. For example, there are *always* interactions between macromolecules and the column matrix.
- As a result, the assumption that for example, standard proteins used to standardise a SEC column have the same hydrodynamic behavior as a sample (or even each other) through SEC column is not correct.

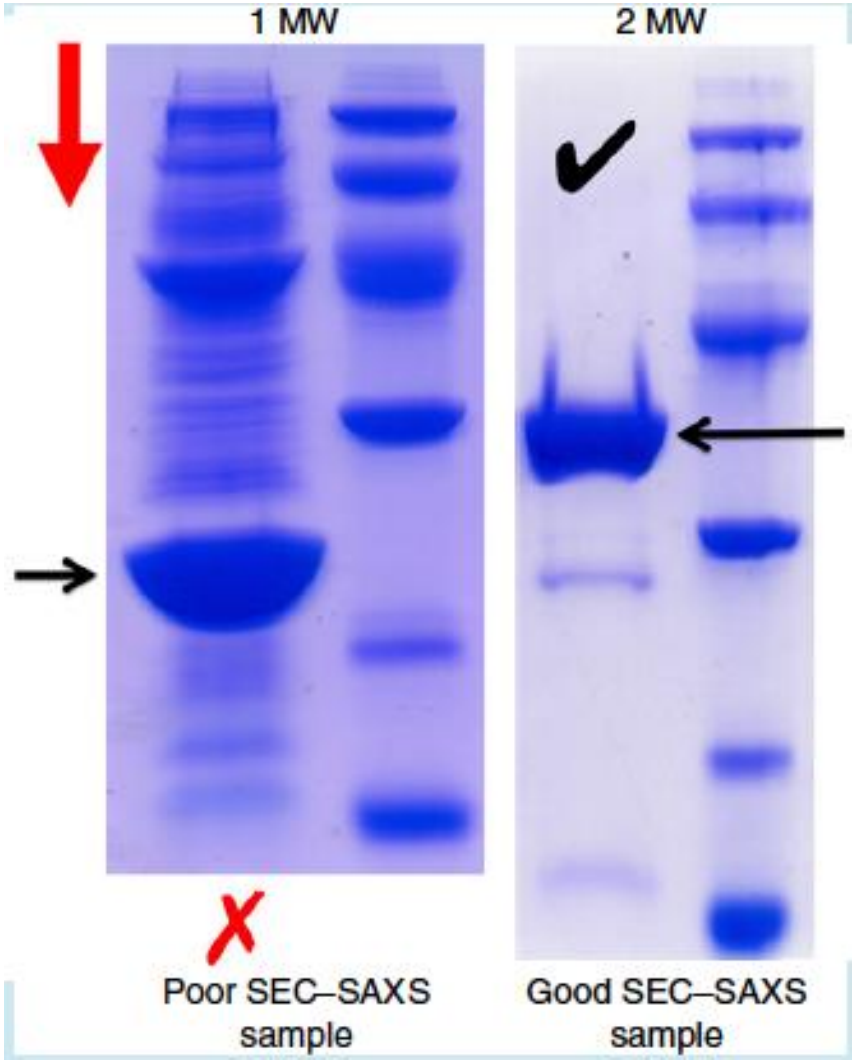
Resolving individual components using SEC

- The SEC resolution is determined by:

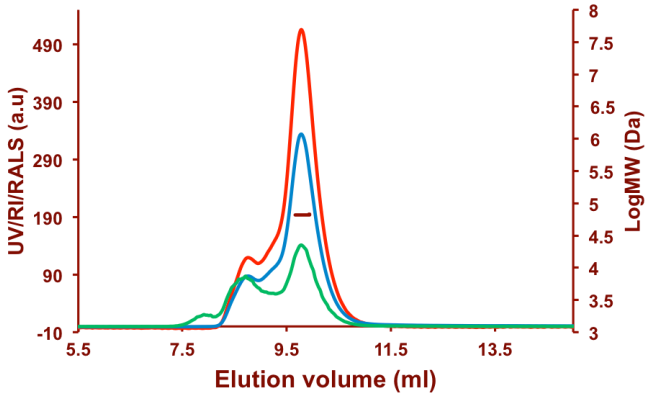
- The physical size of the column.
- The choice of packing matrix (e.g., pore size).
- The sample-load volume.
- The sample flow.
- Solvent conditions and;
- Sample purity.

- To successfully separate sample components using SEC, these parameters should be tested (preferably before a SEC–SAXS experiment). If the column resolution is compromised, i.e., the sample elution peaks ‘run into each other’, then the eluting samples will still be a mixture!

Analytical SEC samples



Column choice is important.

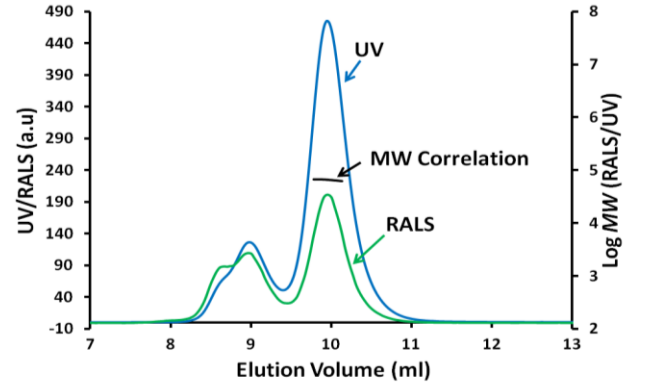


S200 5/150 (2 ml column):

Advantages:

- Fast (15 min)
- Low sample consumption (25-35 µl)

Can only be used as a ‚filter' to remove aggregates. Resolution is too low (e.g., to separate monomer-dimers.)

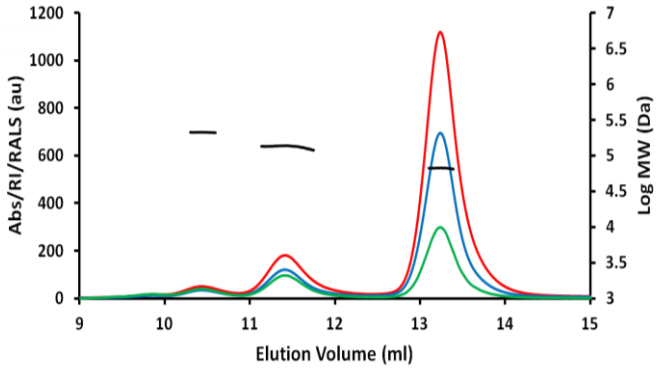


S75 10/300 (24 ml column):

Advantages:

- Excellent resolving power for small monomeric proteins (8-40 kDa.)

Difficult to resolve dimers from monomers and aggregates, especially if dimer MW is close to void volume MW cuttoff (i.e., 70 kDa). 1 hr experiment. Higher sample consumpion (50-75 µl).



S200 Increase (24 ml column):

Advantages:

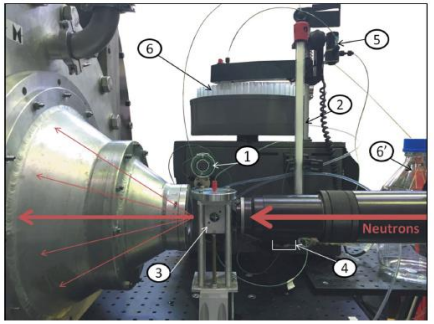
- Resolves a wide MW range (600 kDa to 8 kDa).
- Excellent separation of monomer-dimers
- **<u>Higher pressures, faster flows</u>**. Addition of glycerol to buffers an option (reduce X-ray damage).

Higher sample consumpion (50-75 µl).

SEC-SAS.

- SEC-SAXS systems have been installed at a number of SAXS beam lines, including EMBL-P12 in Hamburg, BioCAT (APS), SWING (Soleil), The Australian Synchrotron, BM29 at the ESRF, the NSRRC Taiwan Light Source SAXS/WAXS beam line (BL23A1).

- SEC-SANS has been demonstrated as feasible at the D22 beamline at the ILL.



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SEC-SANS: size exclusion chromatography combined *in situ* with small-angle neutron scattering[1]

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Obtaining the MW of eluted components from SEC-SAXS

- SEC by itself is pretty good for separation, but is not very reliable as a method to determine the molecular weight of a macromolecule.

- For UV SEC-SAXS: Convent the UV measurements through an elution peak to concentration and then correlate the concentrations to the *I*(0) obtained from SAXS to obtain the MW.

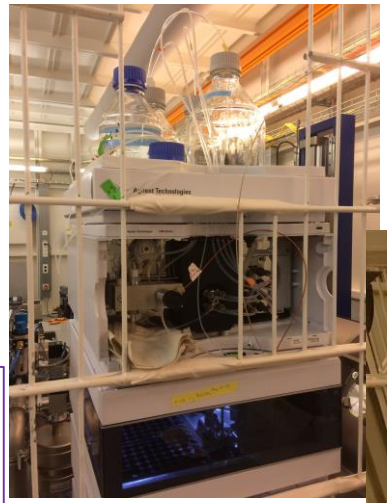
This is not as easy as it sounds. For example band broadening needs to be taken into account (concentration "smearing" effects in the physical tubing connecting the UV to the SAXS instrument) and there is a requirement that components with heterogeneous UV extinction coefficients have been completely separated!

- Use concentration independent methods and compare to MW from UV/I(0)…or…

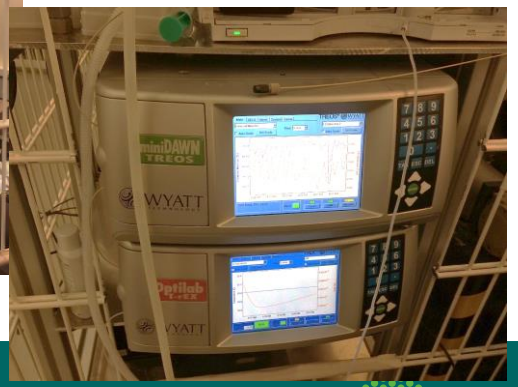
- Combine SEC-SAXS with light scattering methods, RALLS or MALLS, to obtain independent MW estimates of the species eluting form the SEC column.

The Wyatt static and dynamic light scattering system at P12.

- At P12, the analysis of separated components eluting from SEC columns is performed using a Wyatt miniDAWN Treos (3-angle MALLS) with in-bulit QELS plus a Wyatt T-Rex RI instrument.
- The RI is used to evaluate the **sample concentration**
- The MW average data from MALLS can be compared to the MW evaluated from SAXS forward scattering intensities ($I(0)$, in combination with concentration from RI or additional concentration-independent MW estiamte methods.
- The R_h can be compared to R_g from SAXS (to obtain the shape factor).



Agilent
FPLC
HPLC



Molecular weight validation
Hydrodynamic radius, R_h, measurements

Malvern

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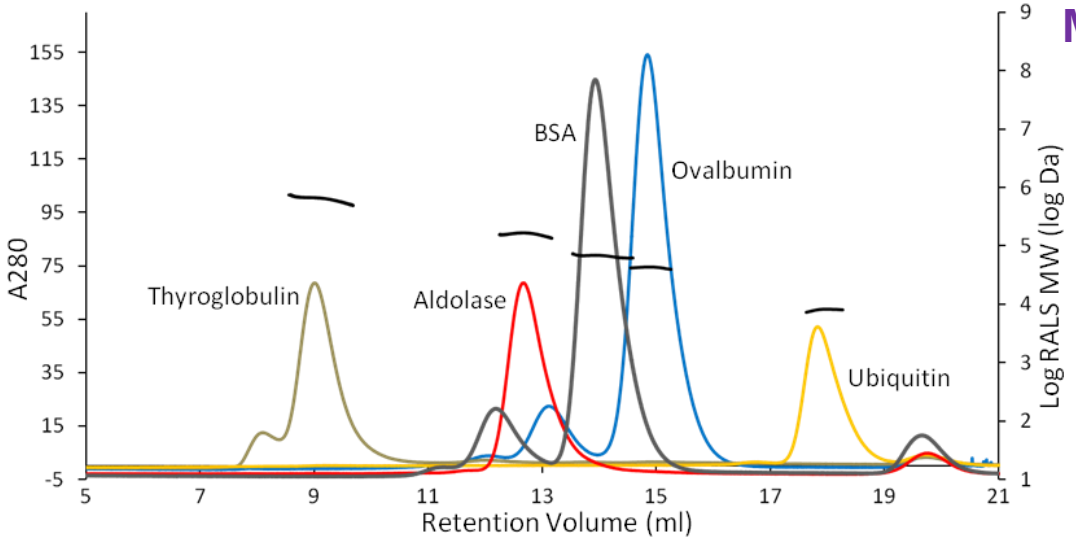
MW from static light scattering.

- The molecular weight average of the eluted components are determined from the correlation between their concentration (*C*), evaluated from the RI and the MALLS intensities.



Retention volume Summary			
Protein	Expected MW (Da)	RALS MW (Da)	Ratio. RALS:expected MW
Ubiquitin	8500	7913	0.93
Ovalbumin (monomer)	43000	42443	0.99
BSA (monomer)	66000	65970	1.00
Aldolase	158000	157929	1.00
Thyroglobulin	669000	638208	0.95

MALLS (R, *q*) = $\boldsymbol{C}(dn/dc)^2\boldsymbol{MW}k_{MALLS}$

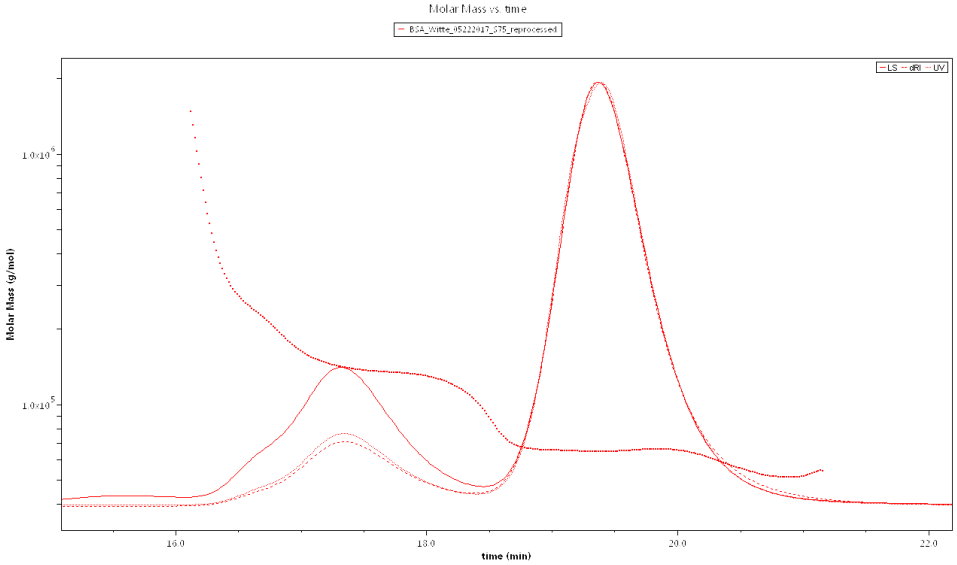
- **RI** = $\boldsymbol{C}(dn/dc)k_{RI}$
- dn/dc is the refractive index increment of unmodified protein, 0.185 $mL.g^{-1}$.
- (Or use UV to determine *C*.
- **UV** = $\boldsymbol{C}\varepsilon k_{UV}$,

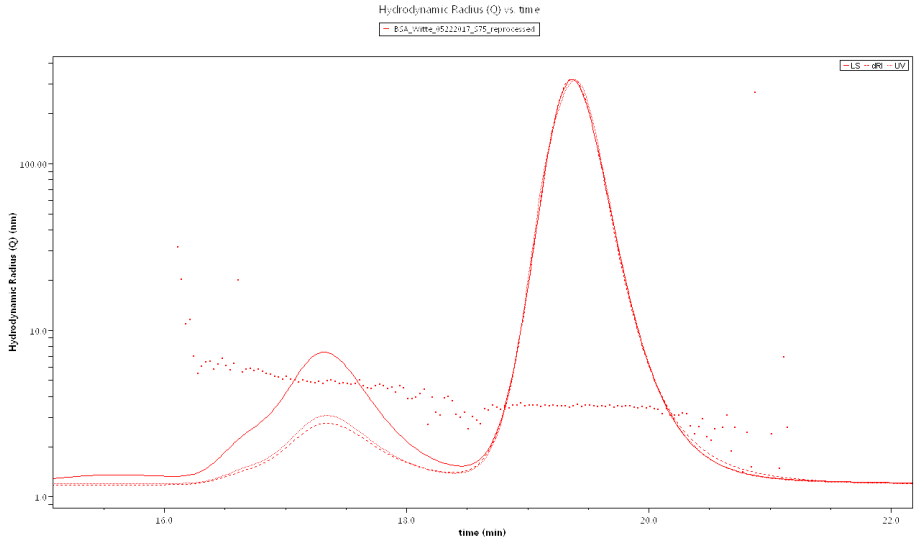
ε is the extinction coefficient (at a chosen wavelength, e.g., 280 nm)).

- k_{RI}, k_{UV} and k_{MALS} are instrument calibration constants, for each detector at angle θ.

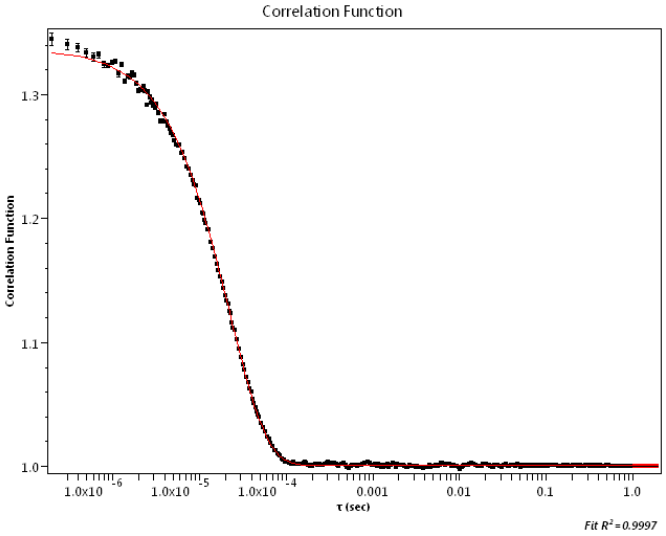
The light scattering MW estimates are independent of elution volume!

Output.





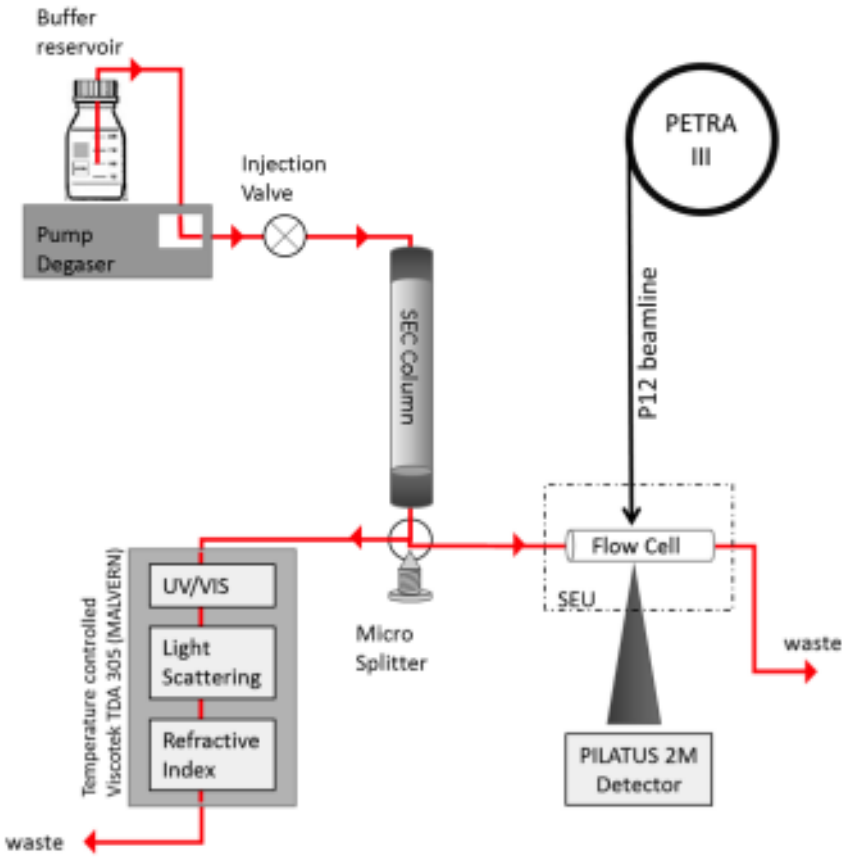
- MW correlations through an elution profile
- R_h correlations through an elution profile.
- DLS auto-correlation function.



M.O.S.E.S.

Microsplitting for **O**nline **S**eparation, **E**xtended **C**haracterization and **S**AXS analysis.

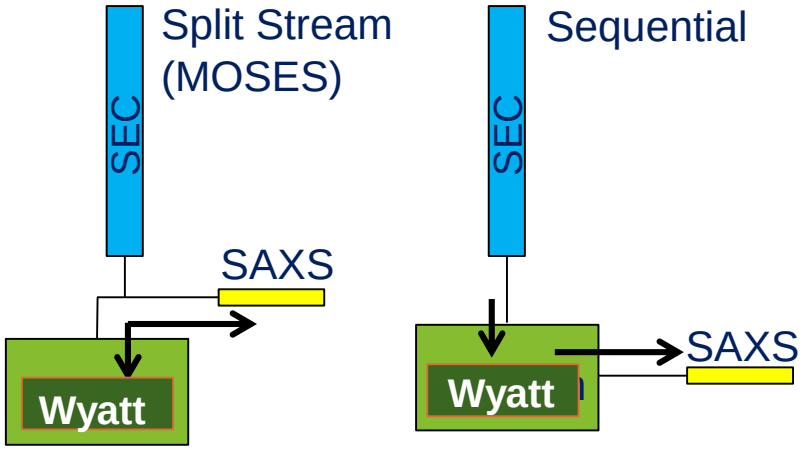
- Sample flow is split between MALLS and SAXS beam line.
- SAXS sample avoids RI detector (25 °C).
- Reduced band broadening.

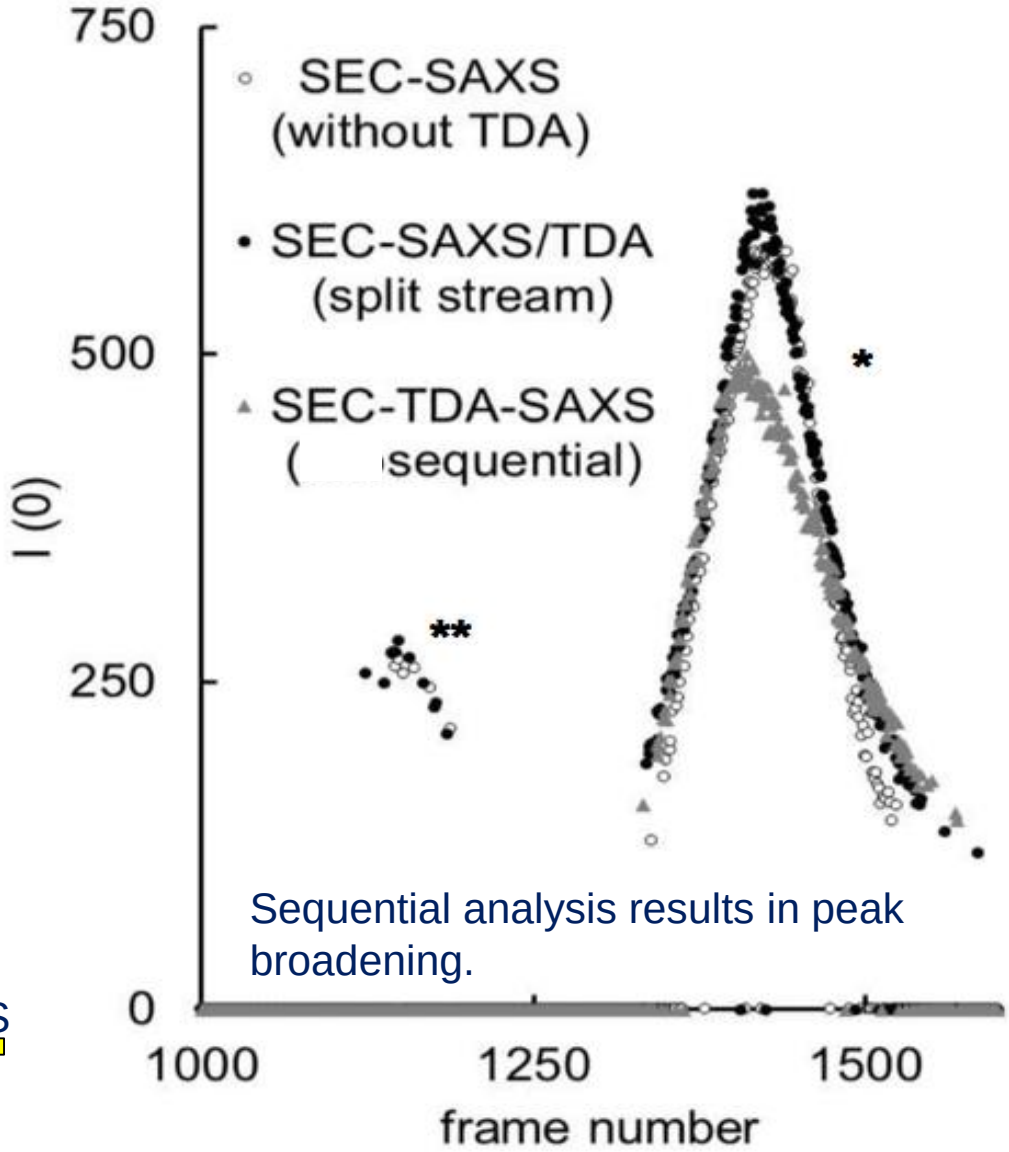


Graewert et al. (2015) *Scientific Reports,* 5:10734 doi:10.1038 /srep10734

More importantly using a split-flow:

The SEC separation (i.e., resolution) is maintained through to the SAXS beam line.

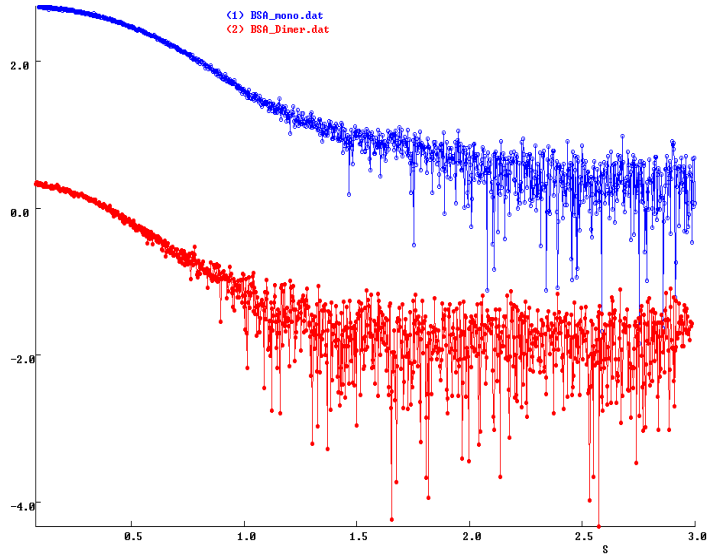




Graewert et al. (2015) *Scientific Reports*, 5:10734 doi:10.1038 /srep10734

Frame averaging, analysis and MW validation.

Combined averaged SAXS data



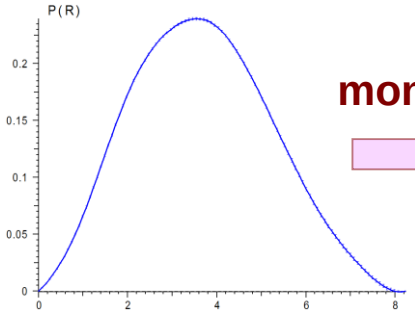
MW: from Malvern and SAXS Porod volume

Molecular Weight Validation

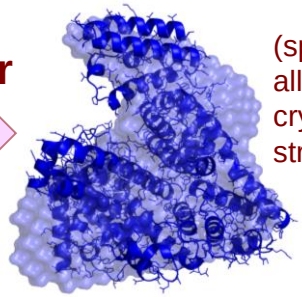
Protein Mixture	Porod Volume (nm^3)	MW- SAXS (kDa)	MW (Malvern) (kDa)
Monomer	110	**65**	**67**
Dimer	207	**122**	**134**

✓

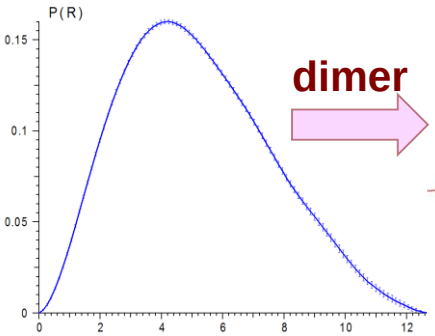
Real-space distance distribution and ab initio modelling



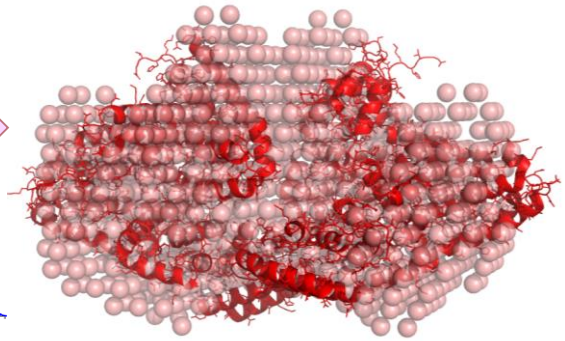
monomer



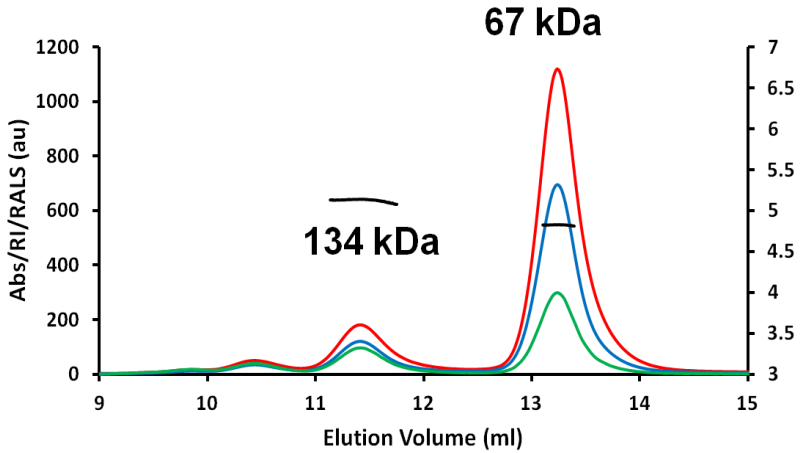
(spatial allignment with crystal structures)



dimer



In-line Malvern output: MW.



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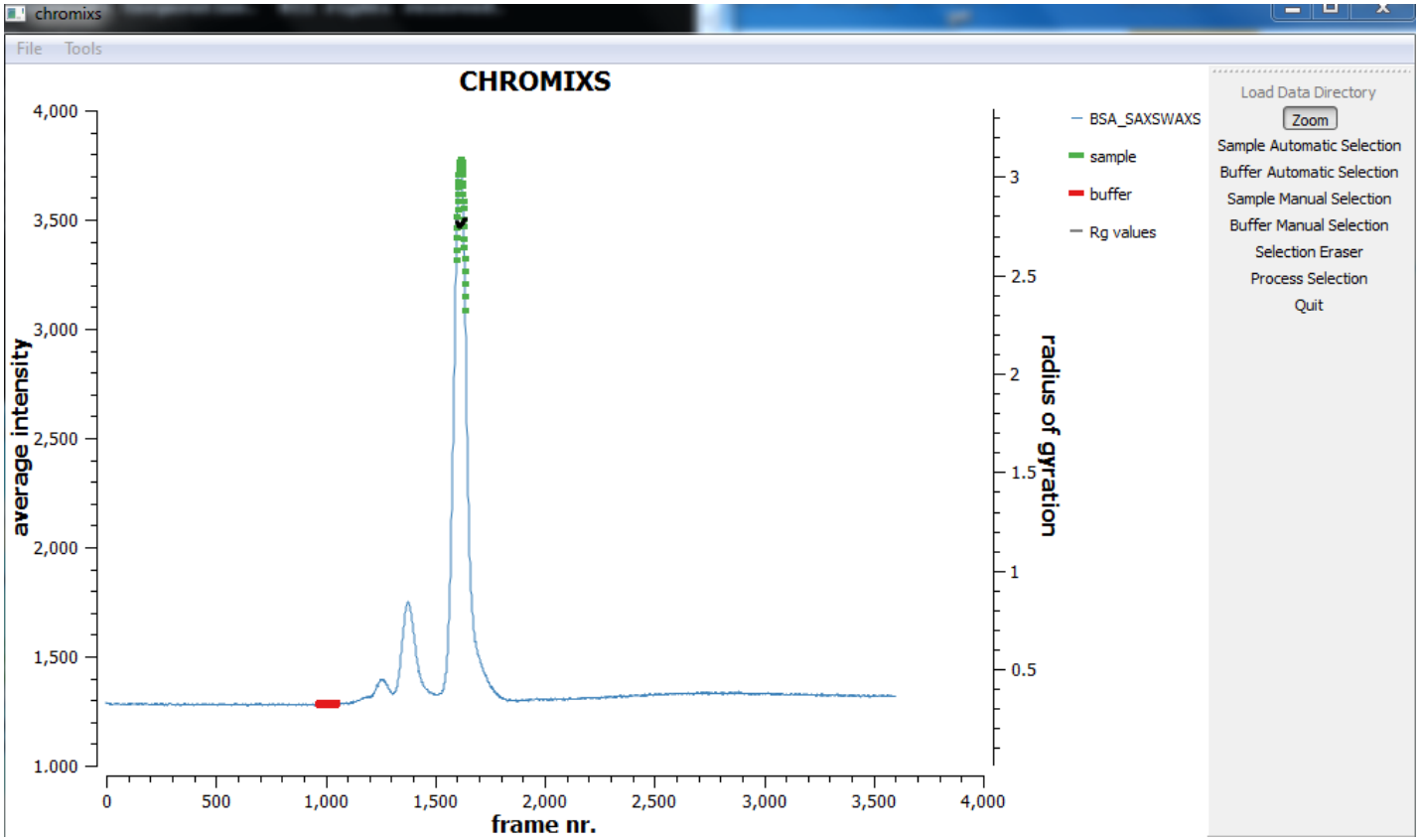
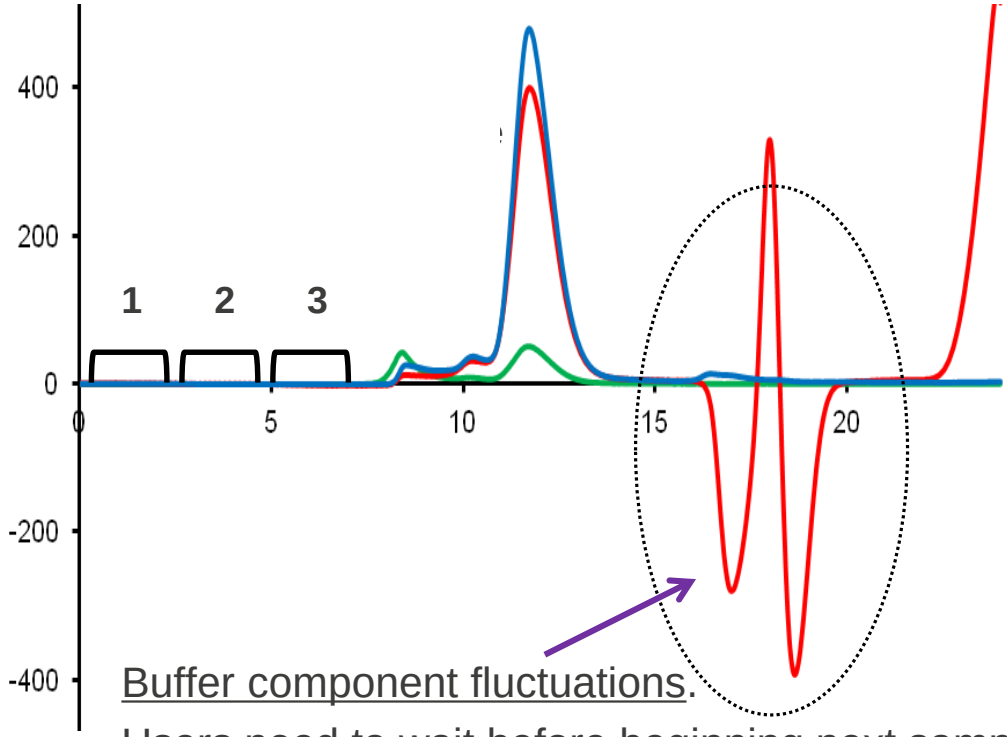
CHROMIXS

- Sasha will demonstrate his very useful and intuitive program CHROMIX for processing SEC-SAXS data after this lecture.



SEC-SAXS: Technical difficulties 1.

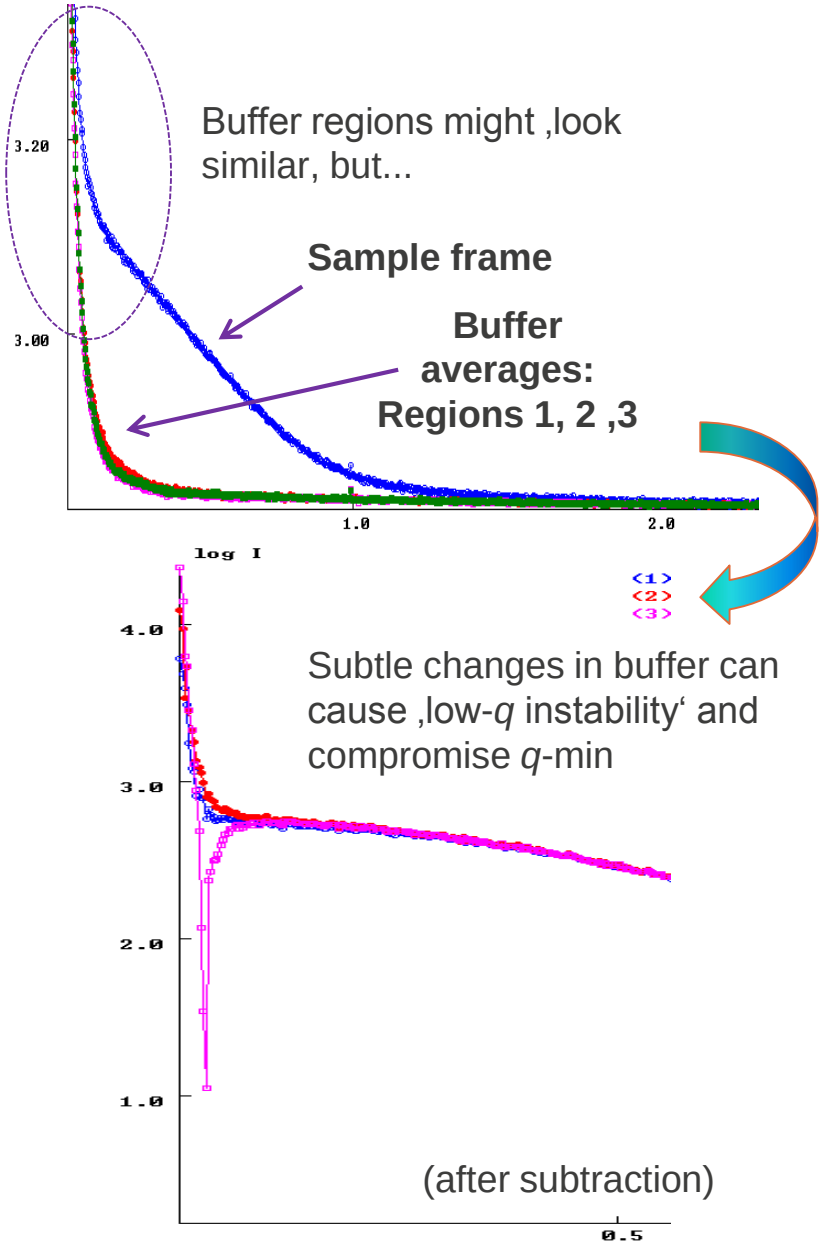
- **Buffer selection.** There is an assumption that the buffer composition does not change significantly through the course of a SEC-SAXS experiment. **This is not necessarily the case.**



Buffer component fluctuations.

Users need to wait before beginning next sample!

COLUMNS MUST BE WELL EQUILIBRATED.



Buffer regions might ‚look similar, but...

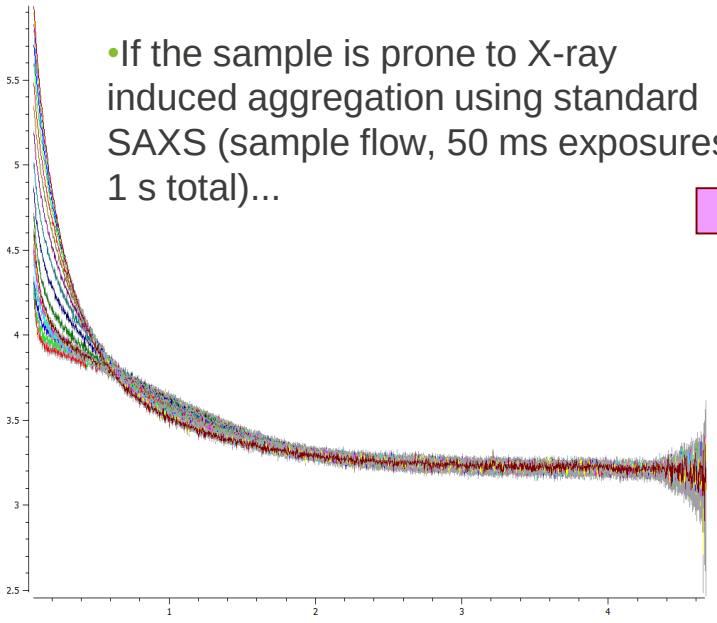
Sample frame

Buffer averages: Regions 1, 2 ,3

Subtle changes in buffer can cause ‚low-*q* instability' and compromise *q*-min

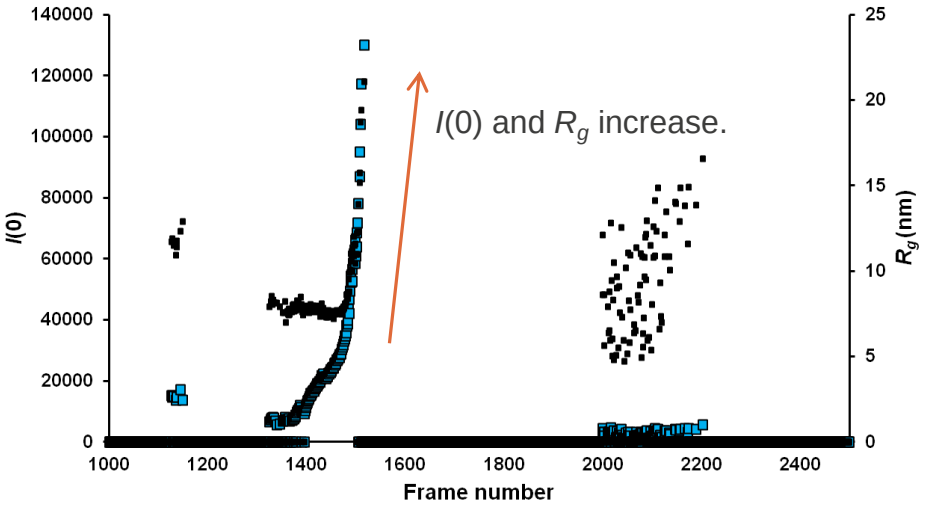
(after subtraction)

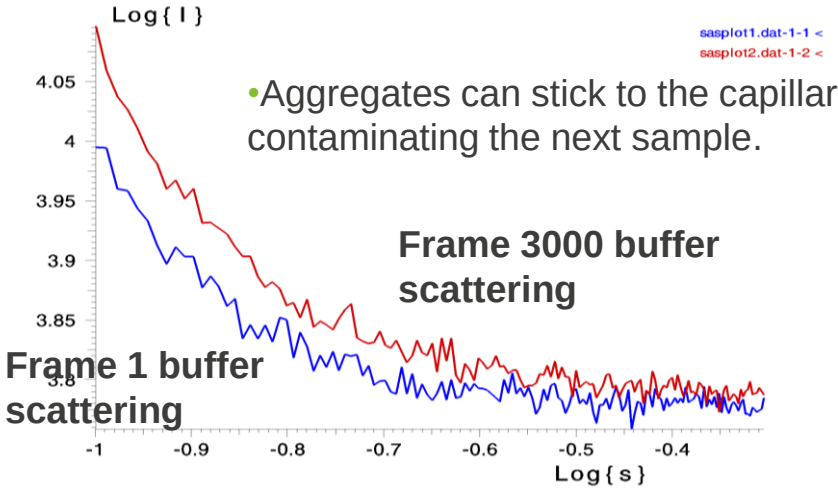
Sample stability (X-ray induced aggregation) needs to be considered.



•If the sample is prone to X-ray induced aggregation using standard SAXS (sample flow, 50 ms exposures, 1 s total)...

...the sample will also aggregate during the continuous flow 1 s exposures of the SEC-SAXS experiment.





•Aggregates can stick to the capillary contaminating the next sample.

The solution..

1) Attenuate the X-ray beam.

2) Add glycerol (3% v/v), DTT, ascorbate, etc (but remember: contrast *vs* agregation *vs* column pressure.)

SEC-SAXS sample and technical considerations

SEC-SAXS – how much sample?

How much sample?

- Higher sample consumption compared to regular batch mode SAXS:

25-75 μl.

7-15 $mg.ml^{-1}$

- The higher concentration reflects the dilution of the sample through a SEC column.

Running buffers.

- Make up an excess of buffer to equilibrate the SEC column before and after the SEC–SAXS experiment. Running buffers need to be very-well filtered and degassed (use 0.1 µm, filters especially when using MALLS).
- Avoid rapid temperature changes of the column and ensure that the buffer and the column are at the same temperature during the equilibration process.
- At high-flux SAXS beam lines, it may be necessary to add solution additives—for example, 2–3% (vol/vol) glycerol, 1–2 mM DTT or 1–2 mM ascorbate—to the SEC running buffer to limit radiation damage. Using Tris or HEPES, ***instead of phosphate***, may also help limit radiation damage.
- It is strongly advised that SAXS data be collected from a small aliquot of sample (e.g., 10–15 µl) using regular SAXS measurements before SEC–SAXS to assess the radiation susceptibility of the sample.

Column Setup.

- The SEC column must be very well equilibrated, typically using 2–8 column volumes of running buffer, before the SEC–SAXS measurement. Extensive column equilibration is required in order to increase the chances of measuring SAXS data corresponding to the matched solvent required for correct background subtraction.
- Note: a stable UV absorption baseline recorded from the buffer flowing off the SEC column (e.g., at 280 nm) is not an indication that the column has, in fact, equilibrated.

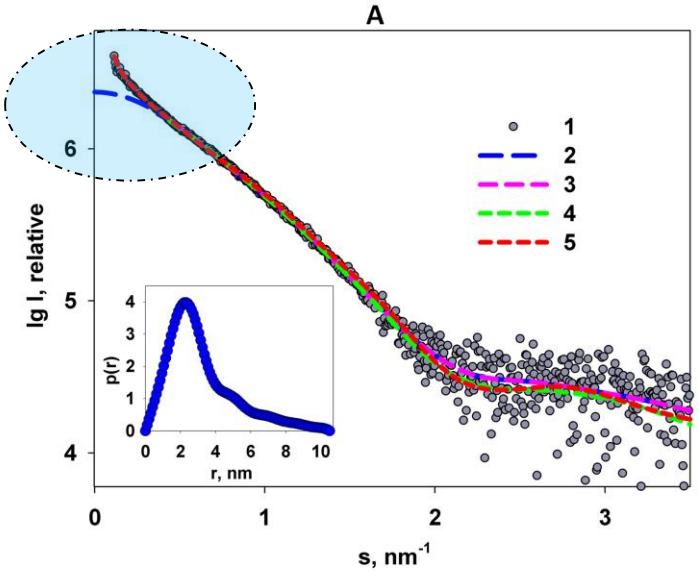
WHY?

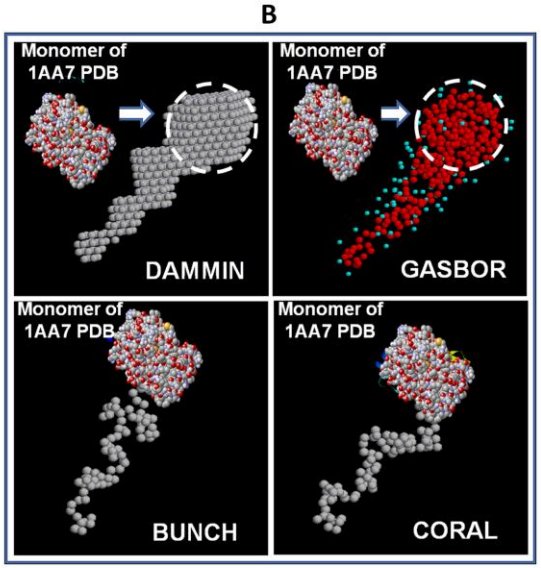
- Both pre- and post-column filtres (e.g., PVDF or PES) are recommended for light scattering experiments.

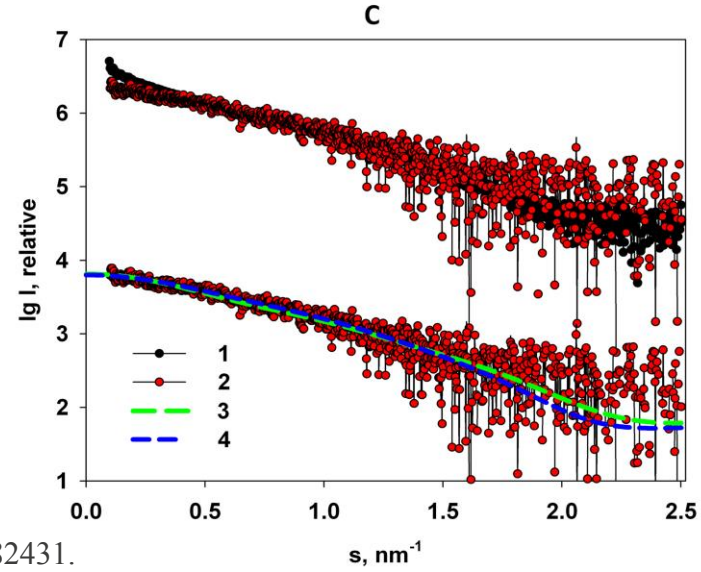
Some Examples

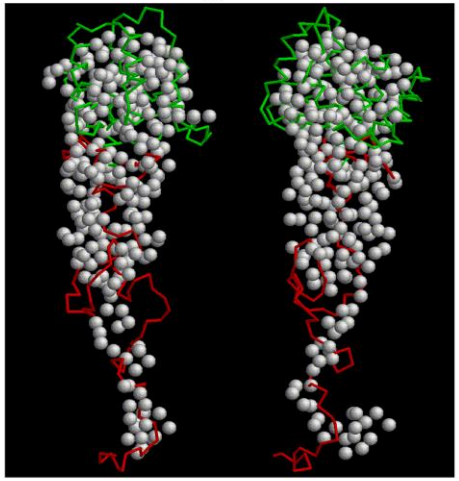
Practical Example: Influenza virus matrix protein M1.

- The M1 protein from influenza virus forms persistent clusters, irrespective of protein concentration.
- Difficult to model monomeric fraction (requires data manipulation, low-*q* removal.)
- SEC-SAXS used to isolate monomeric M1 particles and obtain monomer scattering data.









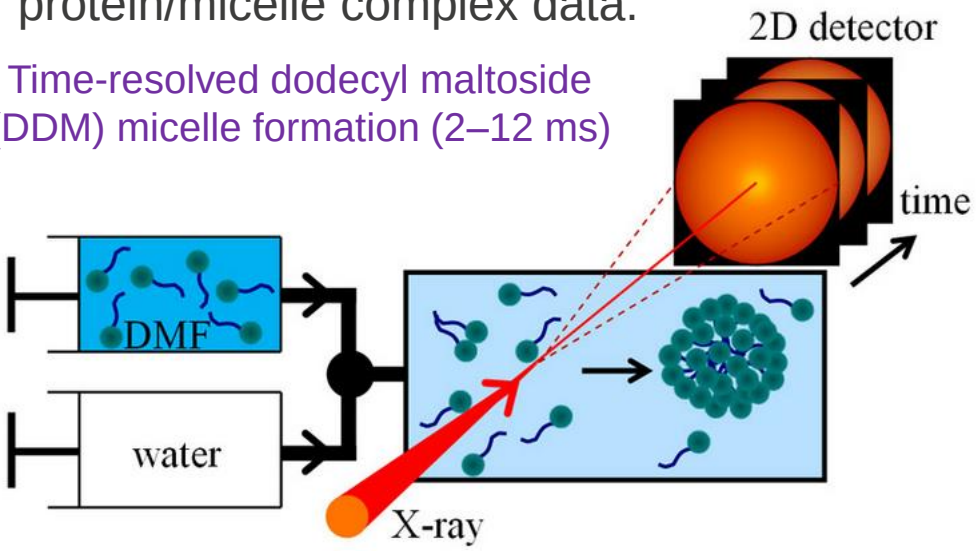
Shtykova et al., (2013) PLoS ONE 8(12):e82431.

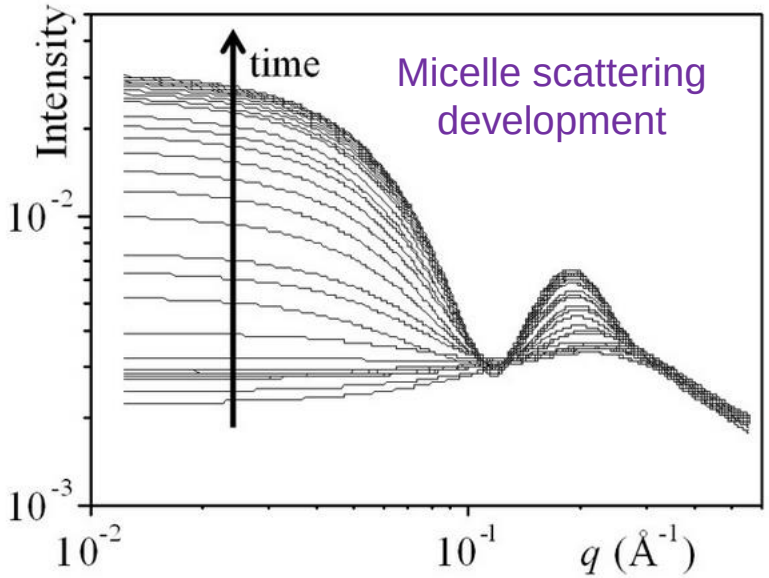
Membrane proteins and the issue with detergents.

- Membrane proteins are often solubilized in detergents. Samples are often mixtures consisting of protein-loaded micelles and empty micelles (unknown concentration; polydisperse.)

- For *regular* SAXS measurements the detergent micelles will scatter strongly making buffer subtraction almost impossible. It is necessary to subtract the empty micelle scattering contributions and the solvent scattering intensities to obtain the protein/micelle complex data.

Time-resolved dodecyl maltoside (DDM) micelle formation (2–12 ms)



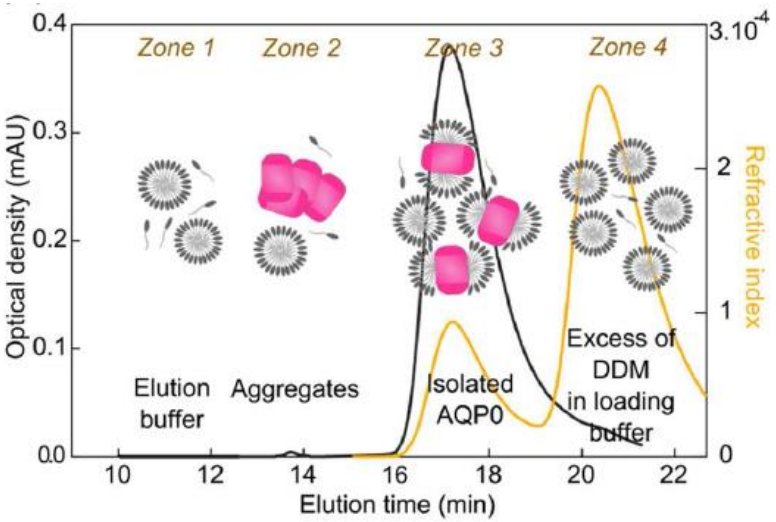


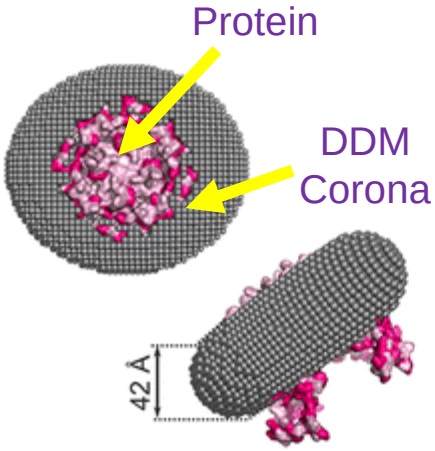
- Work performed at the IDO2 beam line, ESRF, Grenoble.

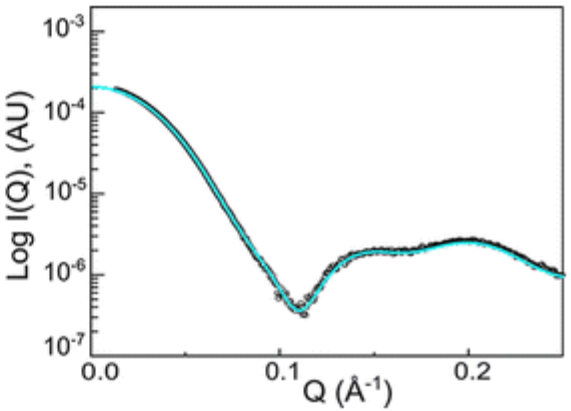
G.V. Jensen, R. Lund, J. Gummel, M. Monkenbusch, T. Narayanan, and J.S. Pedersen (2013) Direct observation of the formation of surfactant micelles under nonisothermal conditions by synchrotron SAXS J. Am. Chem. Soc. 135, 7214–7222.

Practical Example: Protein-detergent mixtures.

- Alice Berthaud's work on Aquaporin-O, an eye lens integral membrane protein.
- Protein embedded in n-dodecyl β-d-maltopyranoside (DDM). The protein-loaded detergent complexes were analysed using SEC-SAXS.
- Bulk empty micelles separated from Aquaporin-O/DDM complex using SEC.







- Work performed at the SWING beam line, Soleil.

Berthaud, A., Manzi, J., Pérez, J. & Mangenot, S. Modeling Detergent Organization around Aquaporin-0 Using Small-Angle X-ray Scattering. J. Am. Chem. Soc. 134, 10080-10088 (2012).

Summary

- SEC-SAXS is an analytical technique used to physically separate the components of mixed samples.
- When combined with MALLS and RI (or UV) measurements, the molecular weights of the eluting species from the SEC column can be validated.
- Extremely useful for removing aggregates, separating oligomers or isolating complexes for SAXS analysis.
- Column choice is important. Requires significant time to set up the column correctly. Proper column equilibration is absolutely necessary (to obtain the matched solvent blank for subtraction.)
- Compared to regular SAXS, SEC-SAXS requires more sample and more time to complete a single experiment (up to 1 hr).
- Careful statistical analysis of data frames is necessary to obtain the correct averaged profile.

SEC–SAXS is ***not*** a ‘cure-all’ technique for every sample and should not be viewed as a purification step but rather as an analytical procedure to be applied as necessary on a case-by-case basis.

If batch mode SAXS is available use it!

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