Sample preparation and characterization around SAXS

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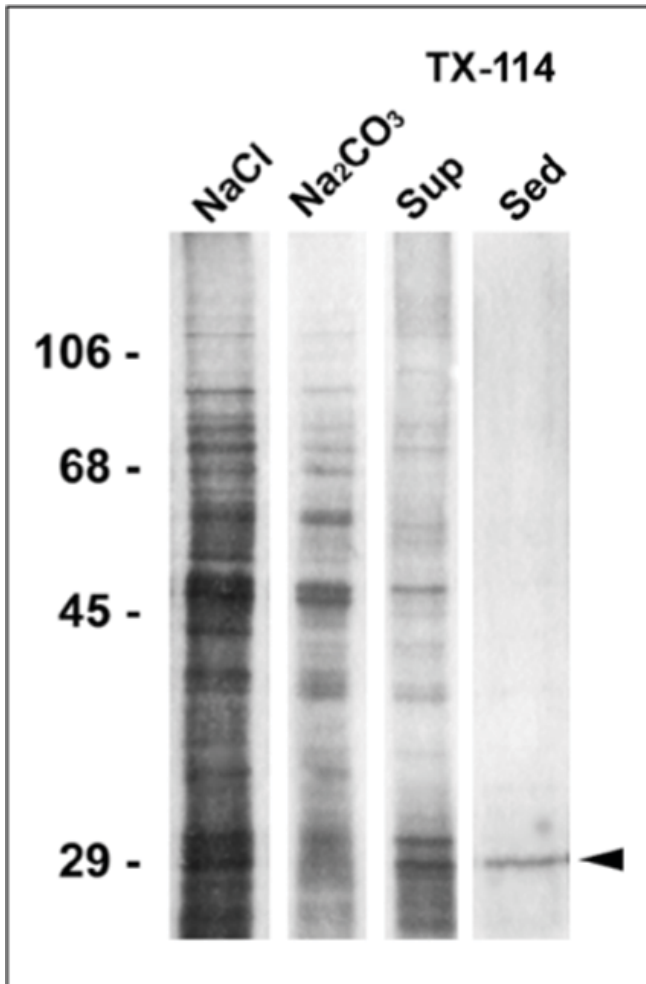
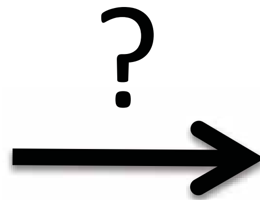
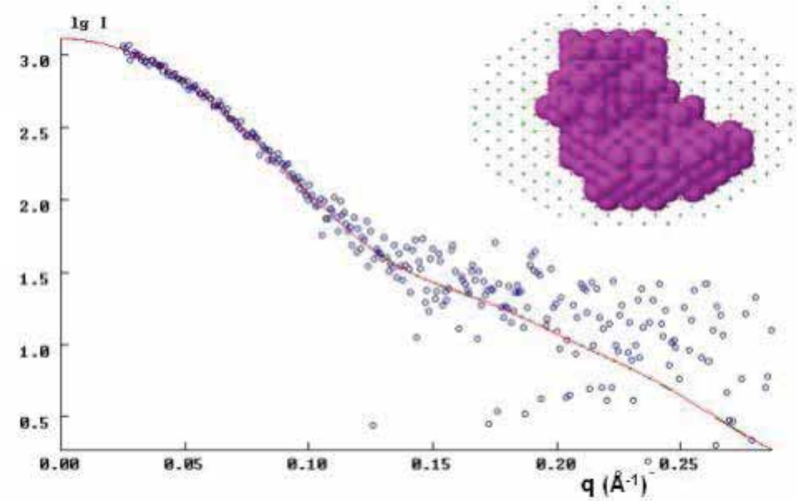
EMBL Hamburg

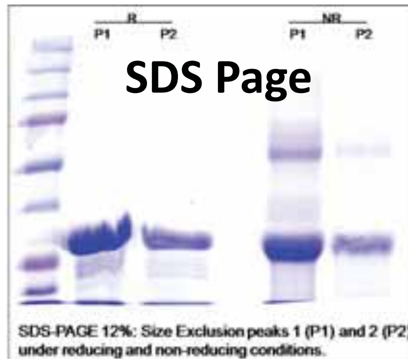
Garbage in

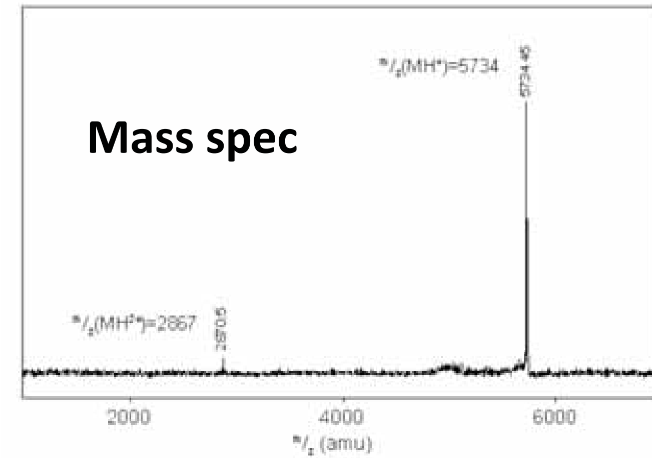


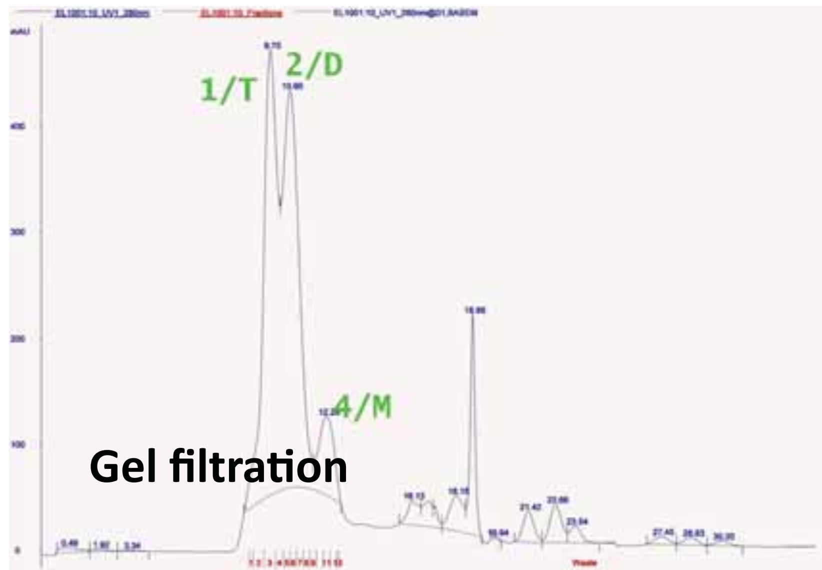


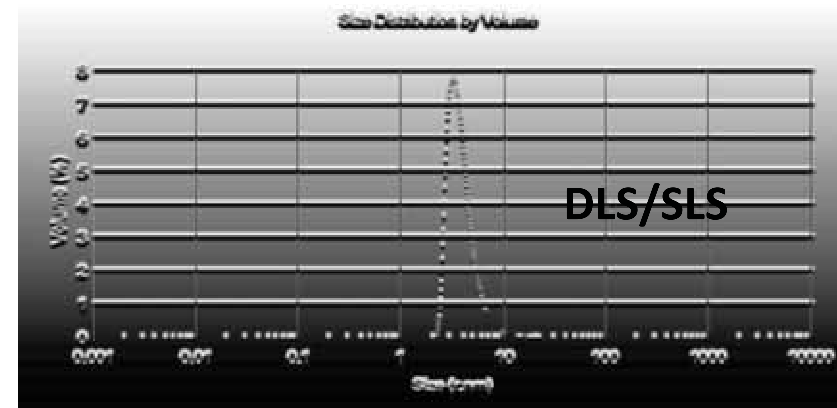


Quality control

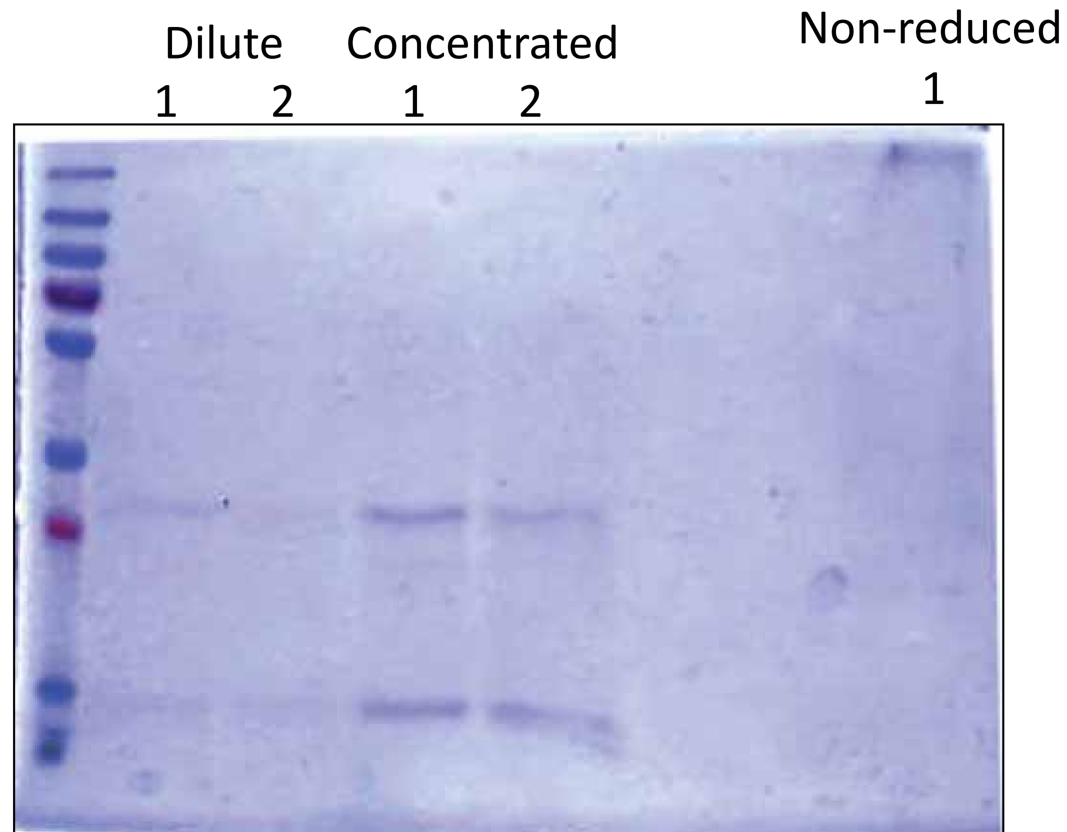




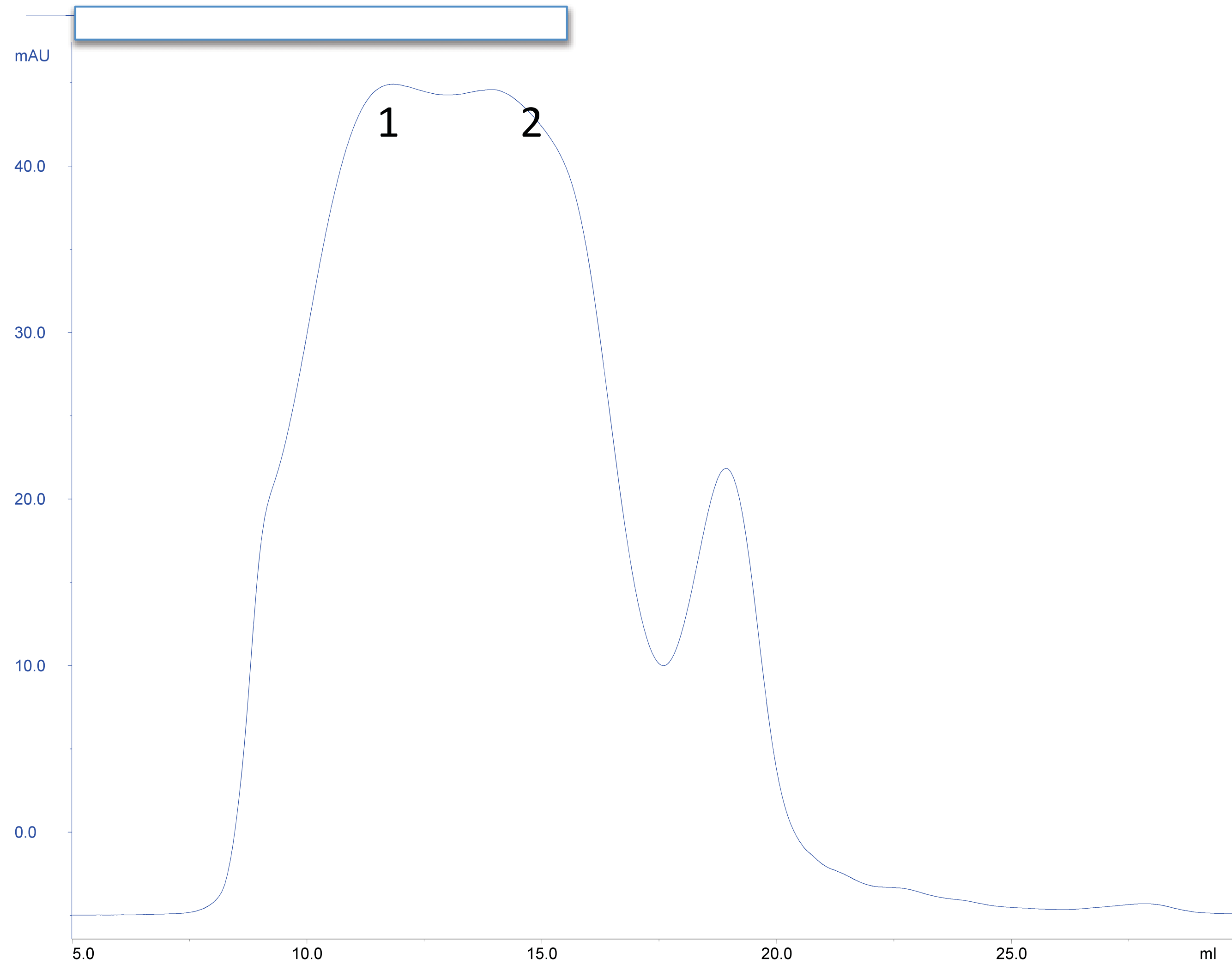




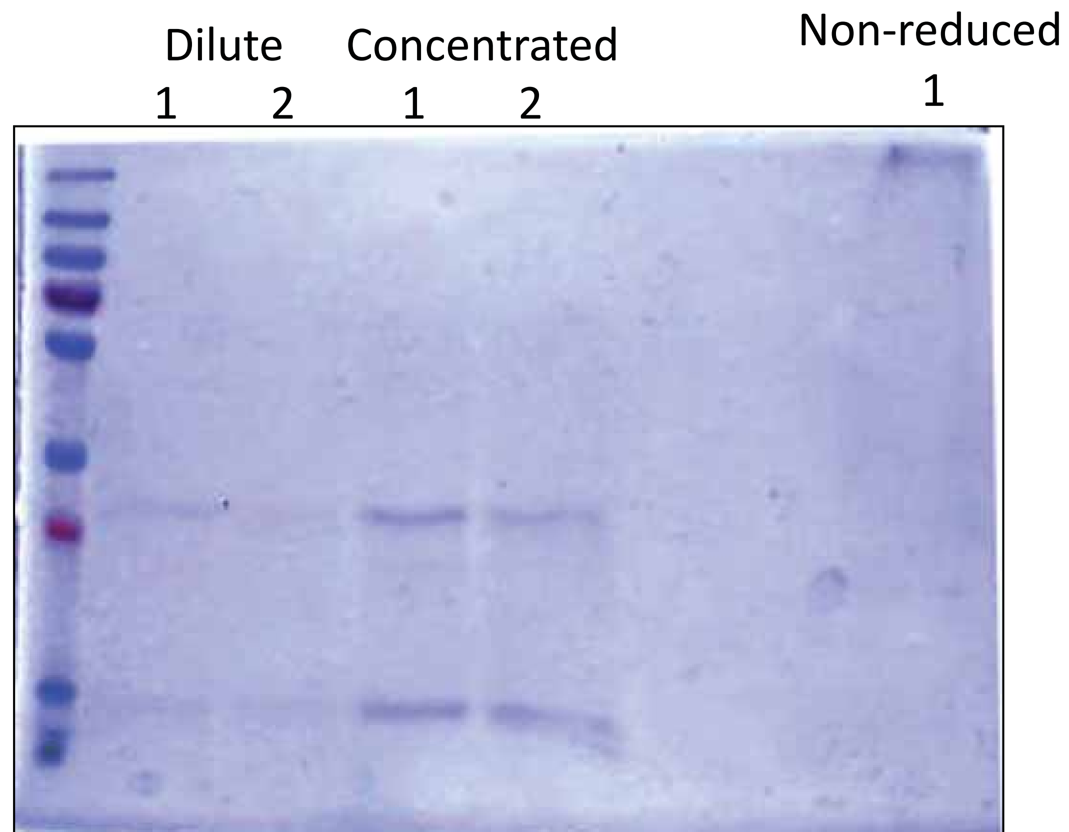
A gel of a heterodimeric receptor



A size exclusion profile



A second look



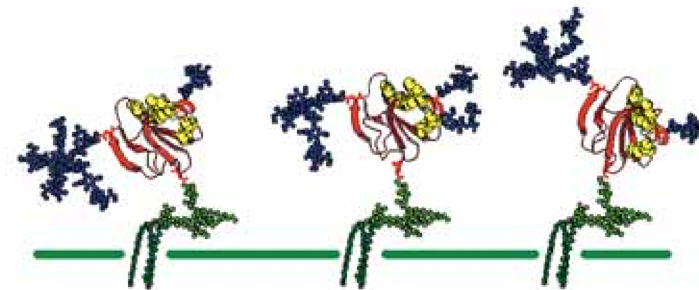
Different gels

- SDS
- SDS non-reduced
- Native
- Iso-electric focussing

- Purity
- Disulphide bonds
- Post-translational modifications:
  - Phosphorylation
  - glycosylation

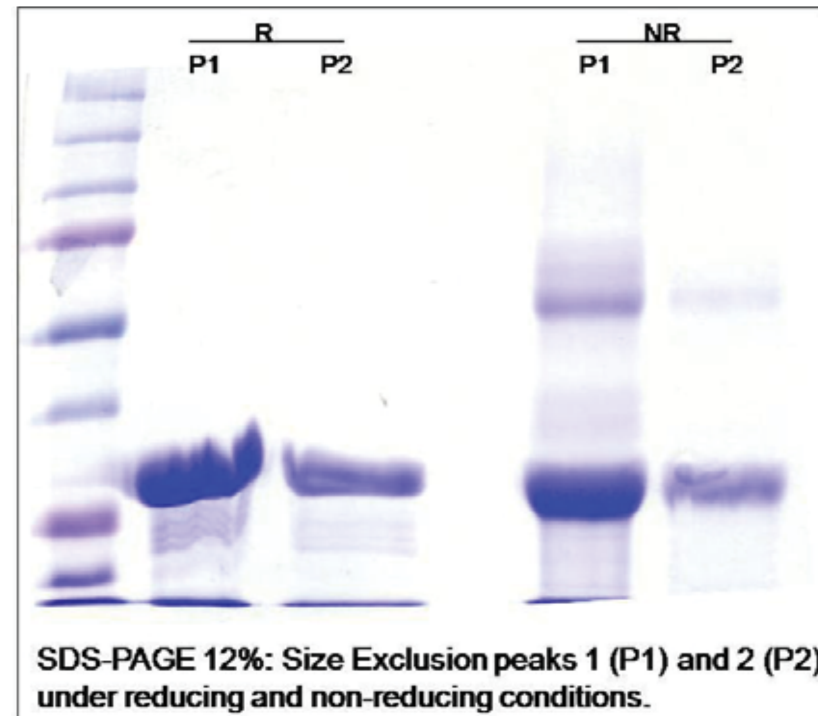
Glycans form a sizeable part of the protein

- Depends on expression system
- In pichia: > 40 % of MW can be glycans!
- Deglycosylate
- Change to HEK/COS/CHO-Lec



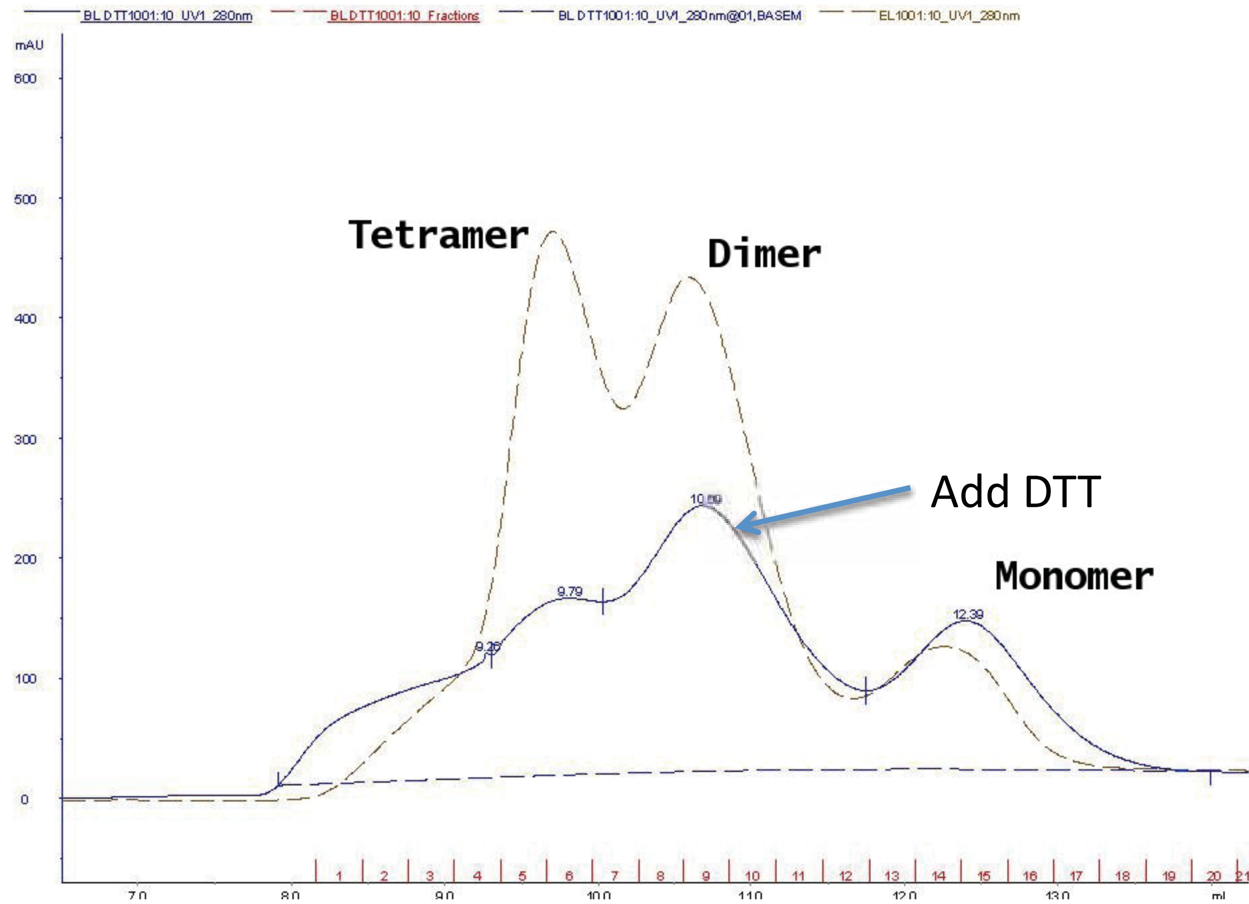
Another capricious sample

7XCYS ORIGINAL SEQUENCE, WITH HIS-TAG

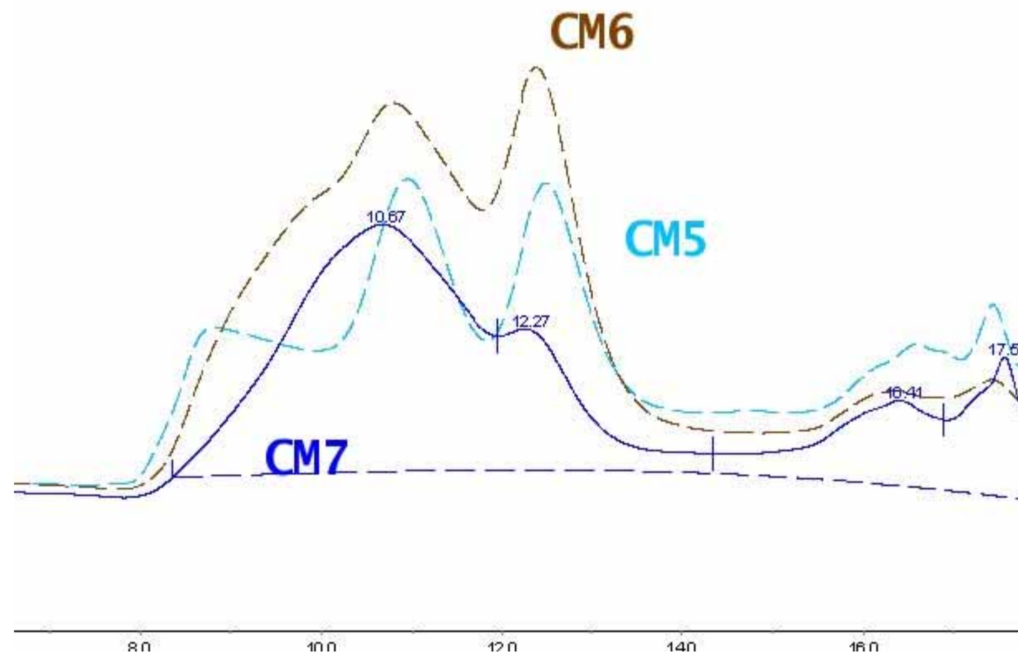


SDS-PAGE 12%: Size Exclusion peaks 1 (P1) and 2 (P2) under reducing and non-reducing conditions.

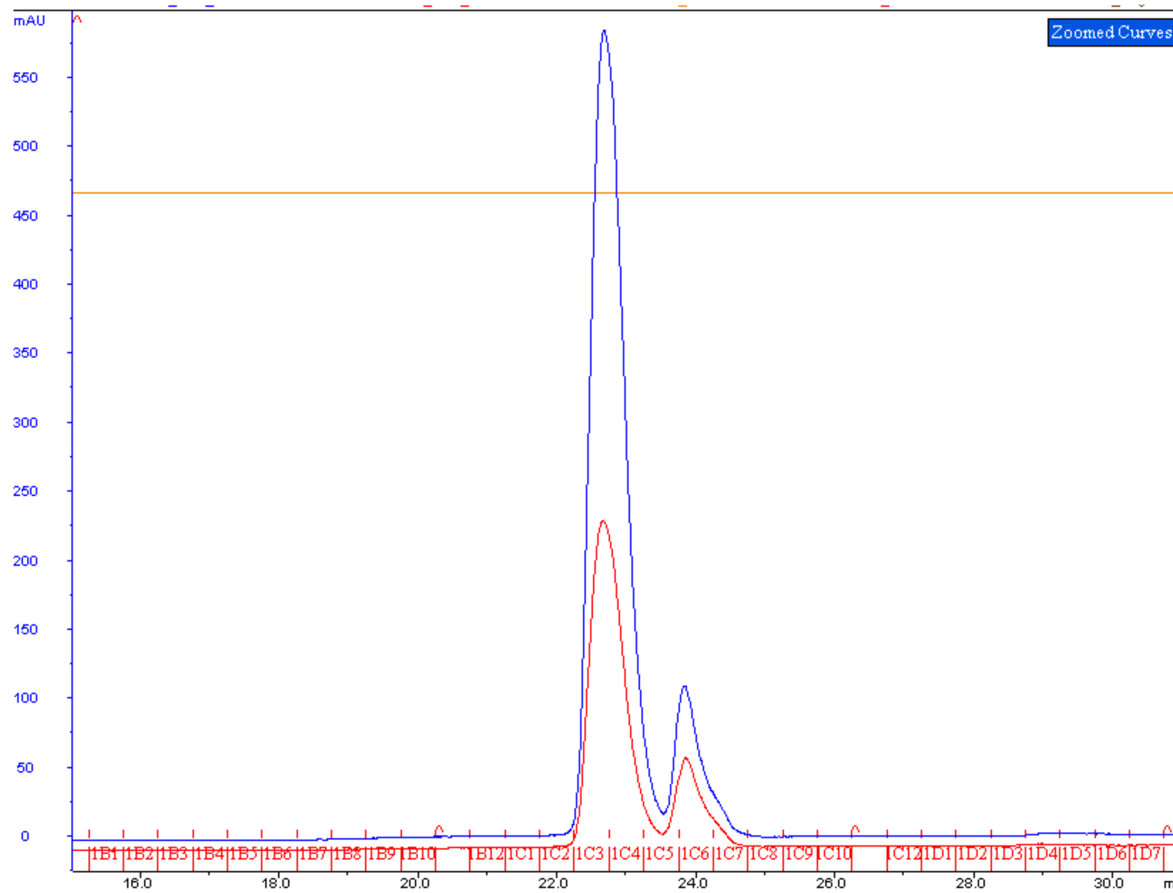
Sizing profile



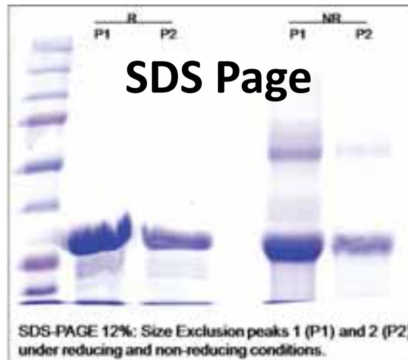
Cysteine point mutations

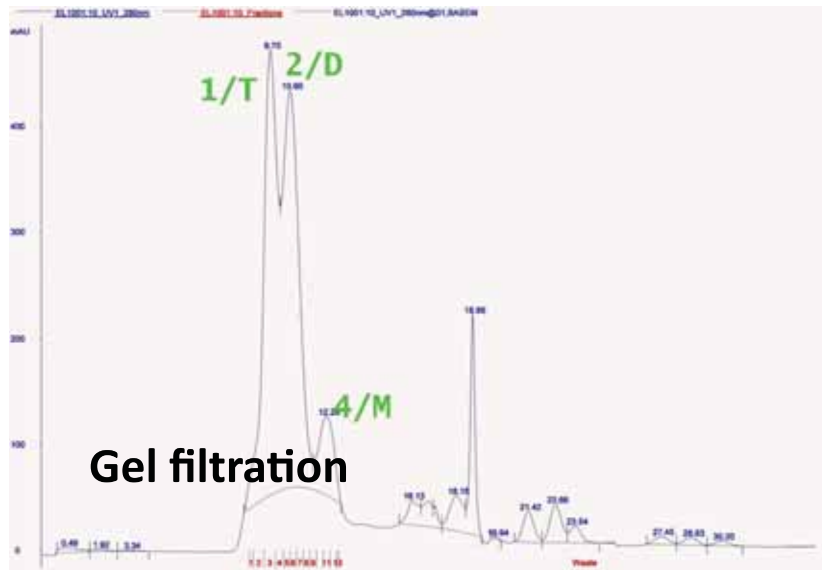


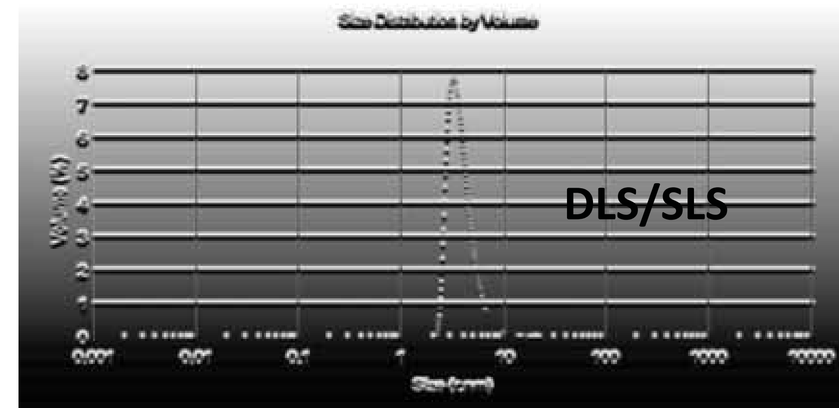
Sizing profile



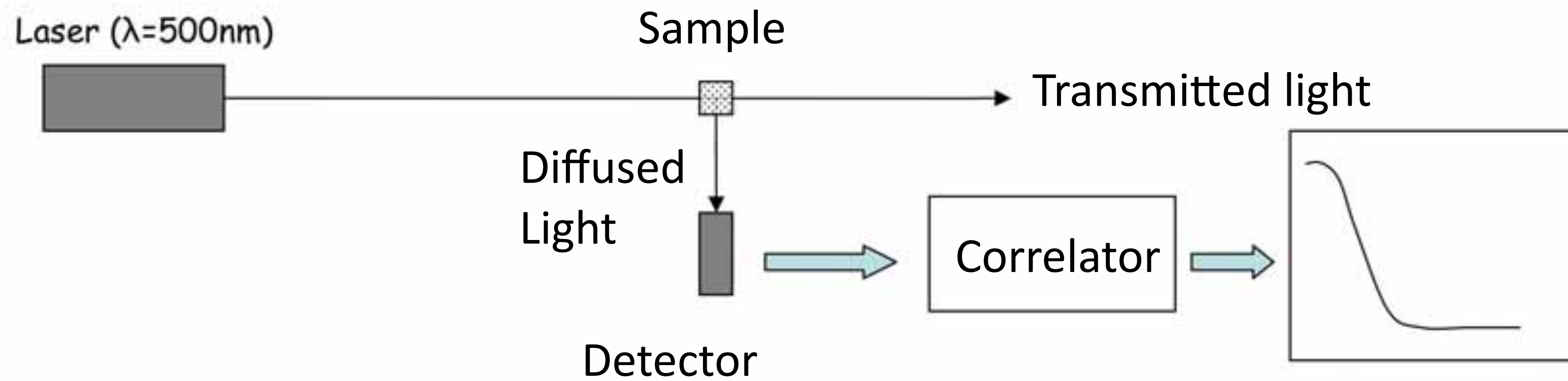
Quality control



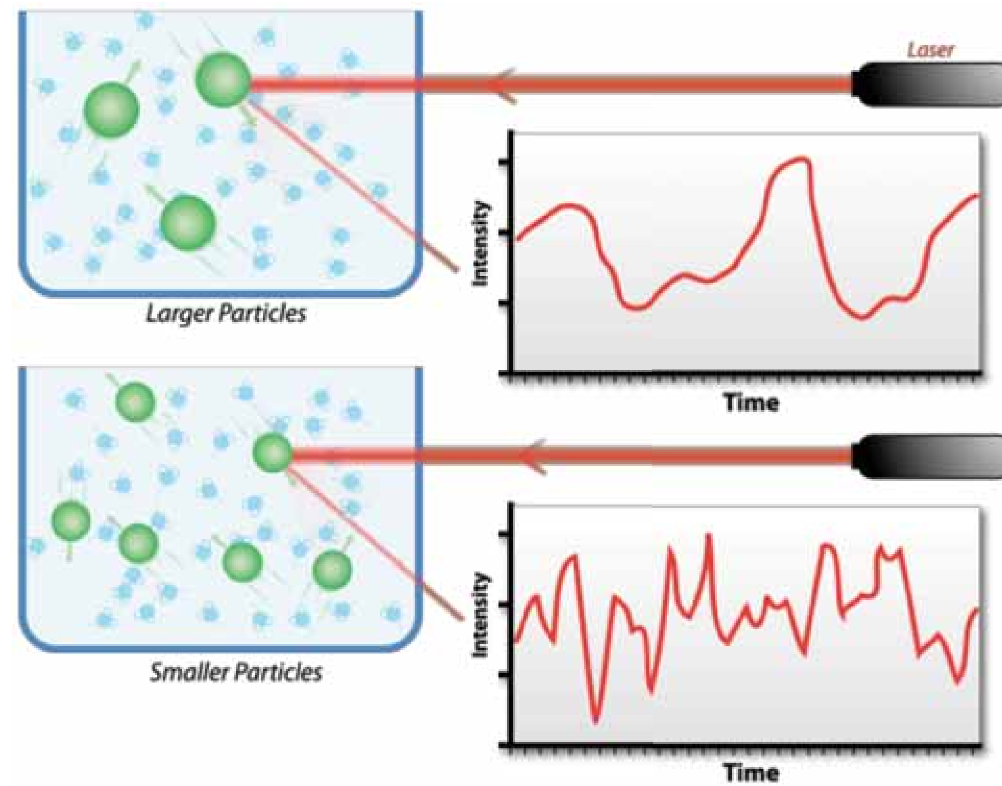




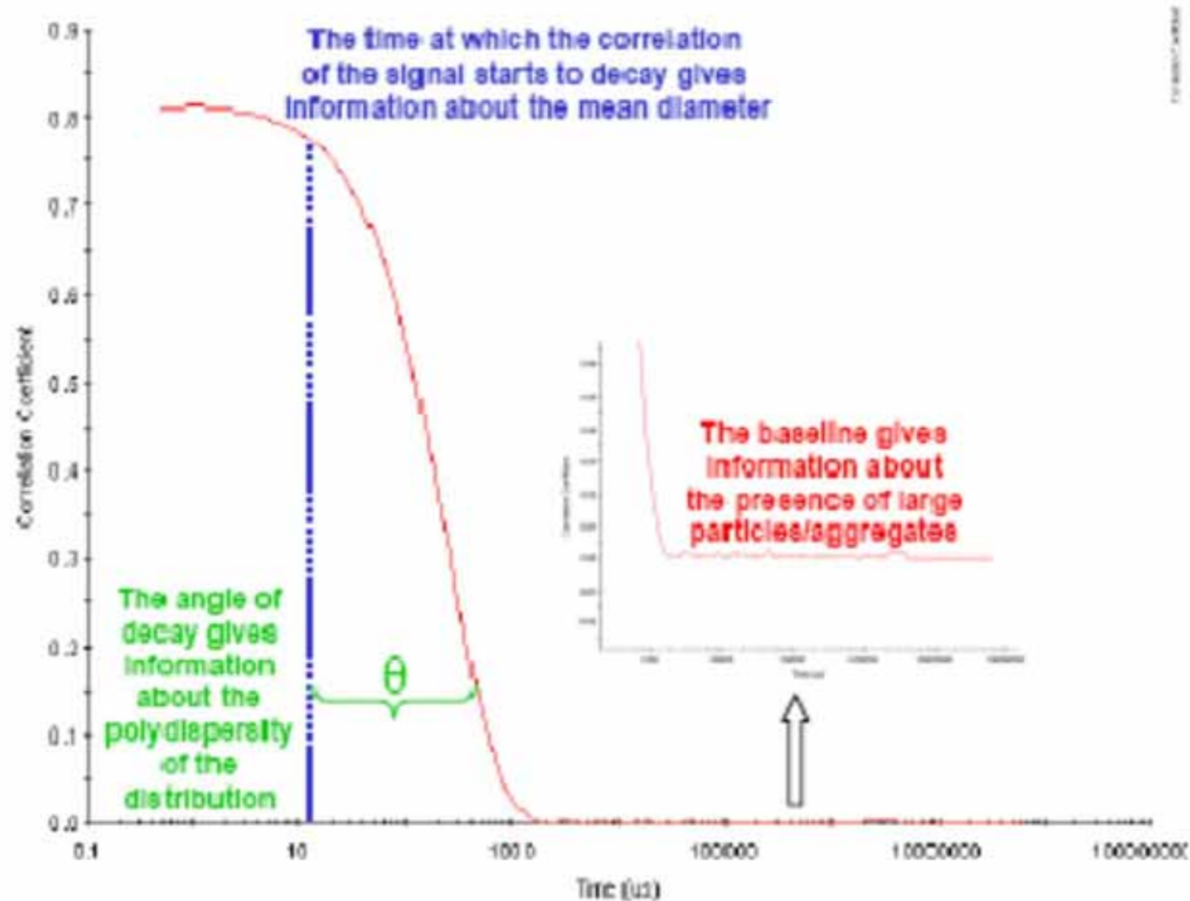
Dynamic Light Scattering scheme



Dynamic light scattering



Information from the correlation curve



Stokes-Einstein relation

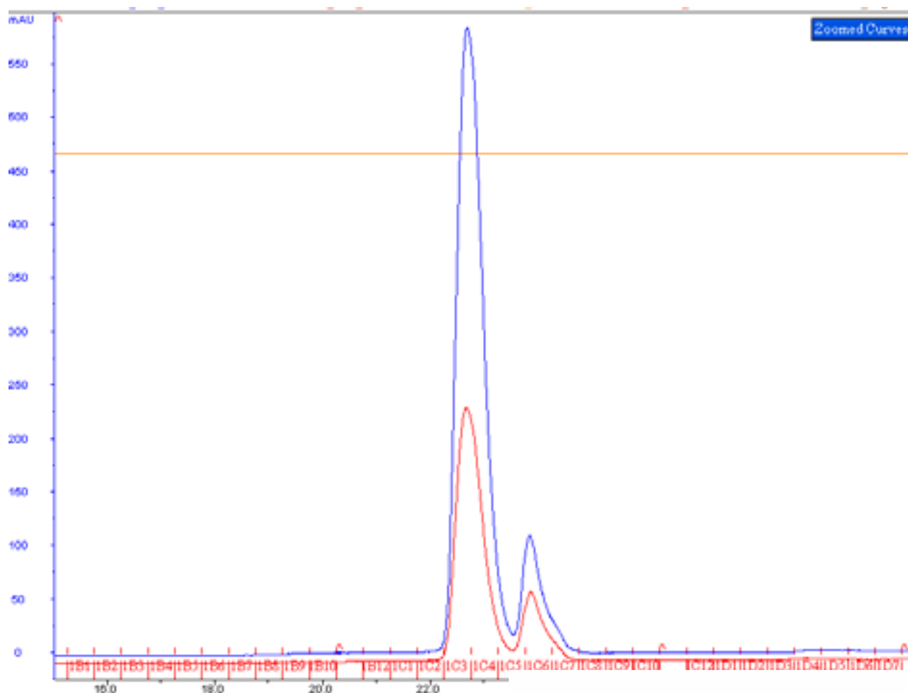
- D = Diffusion coefficient
- k = Boltzmann's coefficient
- T = Temperature
- η = Viscosity
- R = hydrodynamic radius

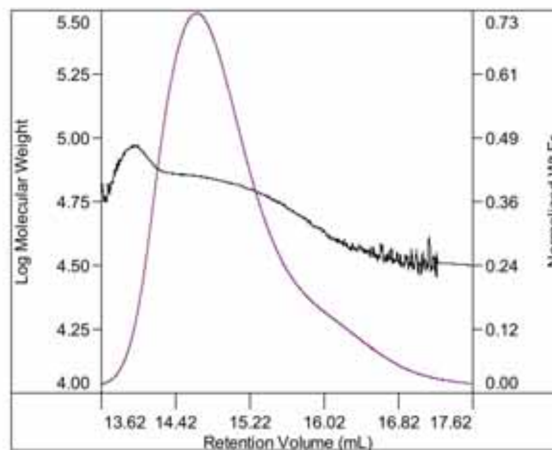
$$D = \frac{kT}{6\pi\eta R}$$

Static Light Scattering

Courtesy Bernd Tartsch, Malvern Instruments

Sizing profile, SLS twist

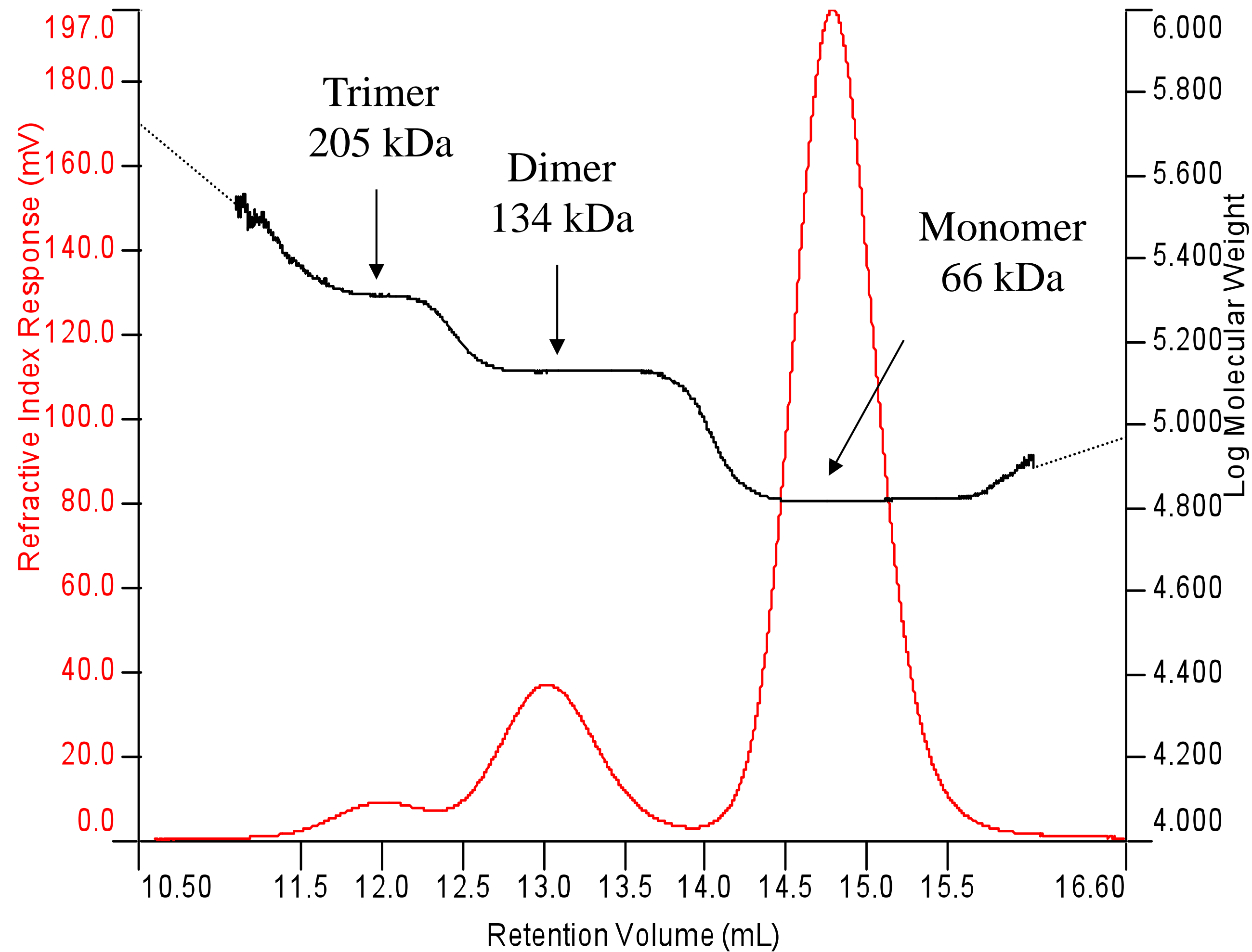




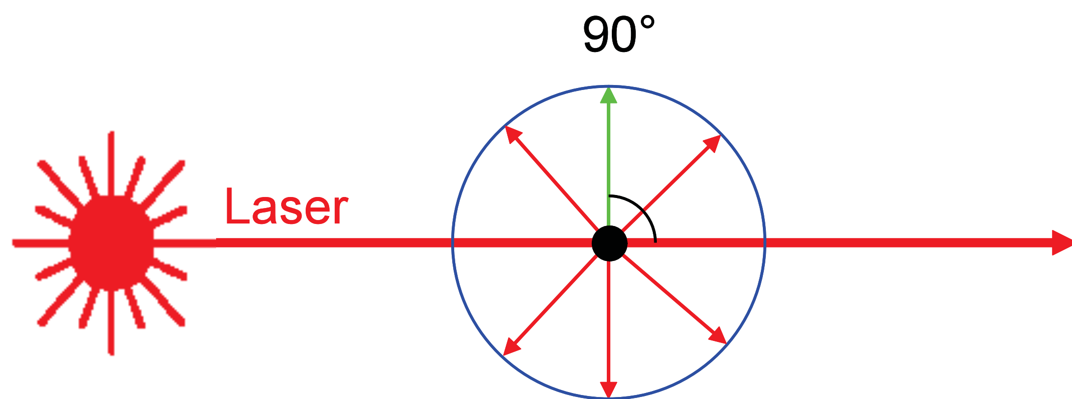
Peak	1		
Mw (Daltons)	64.151		
Mw/Mn	1.055		
IV (dl/g)	0.0434		
Rh (nm)	3.50		
Wt Fr (Peak)	1.0000		

ID	ctp1l
Solvent	20mM HEPES, 150mM NaCl
Flow Rate	0.5000
Conc (mg/ml)	2.0079
Inj Vol (ul)	100.0

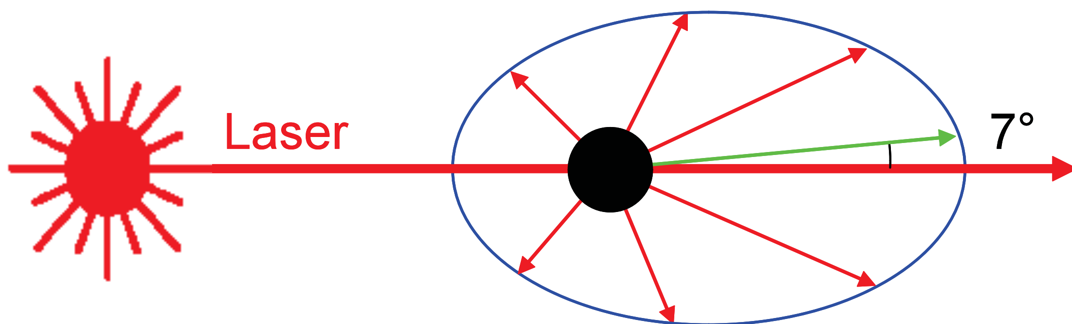
BSA – Molecular Weight



Angular Dependence

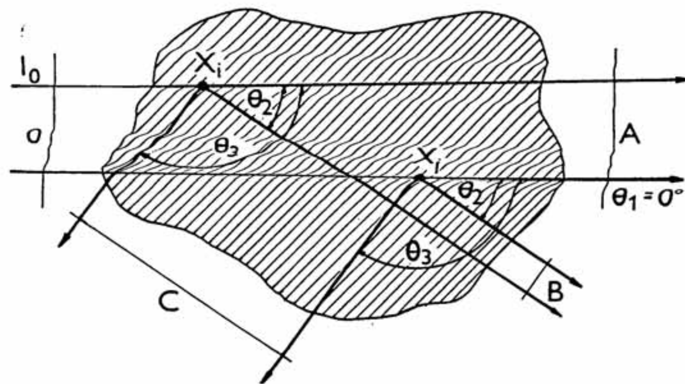


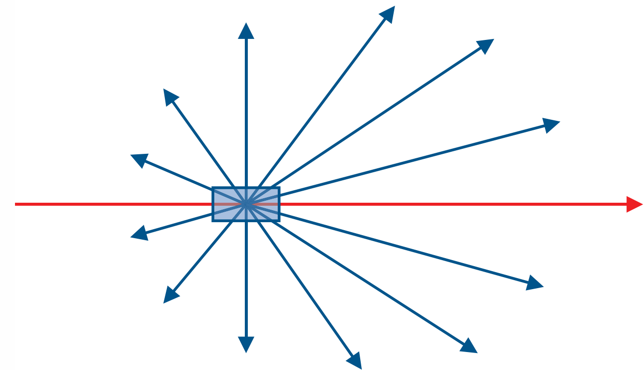
small molecules
radius < 15 nm
$P_\theta = 1$ for all θ



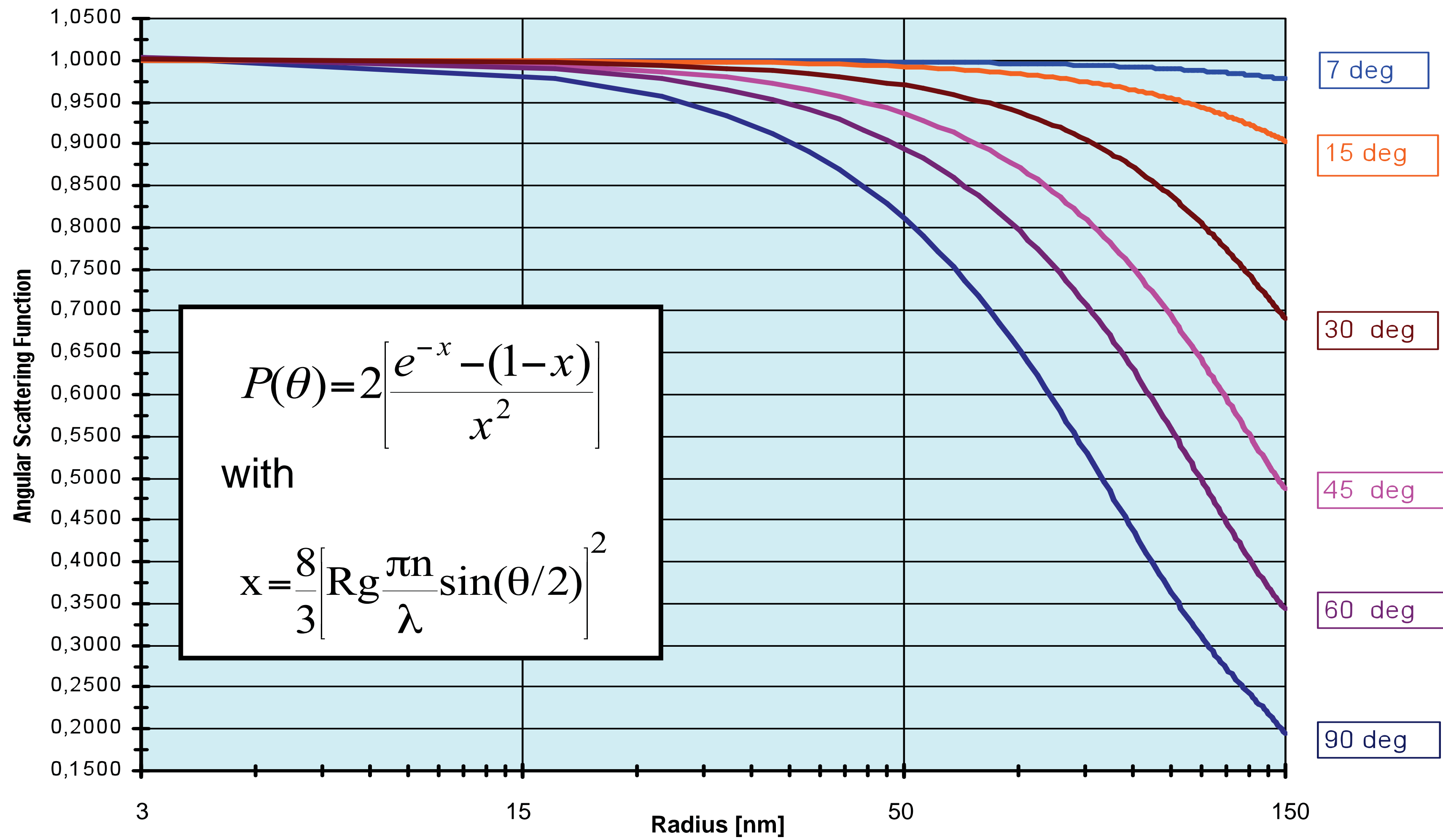
large molecules
radius > 15 nm $P_\theta = 1$ for $\theta = 0°$

Intramolecular interference produces a disymmetry in the scattered light.



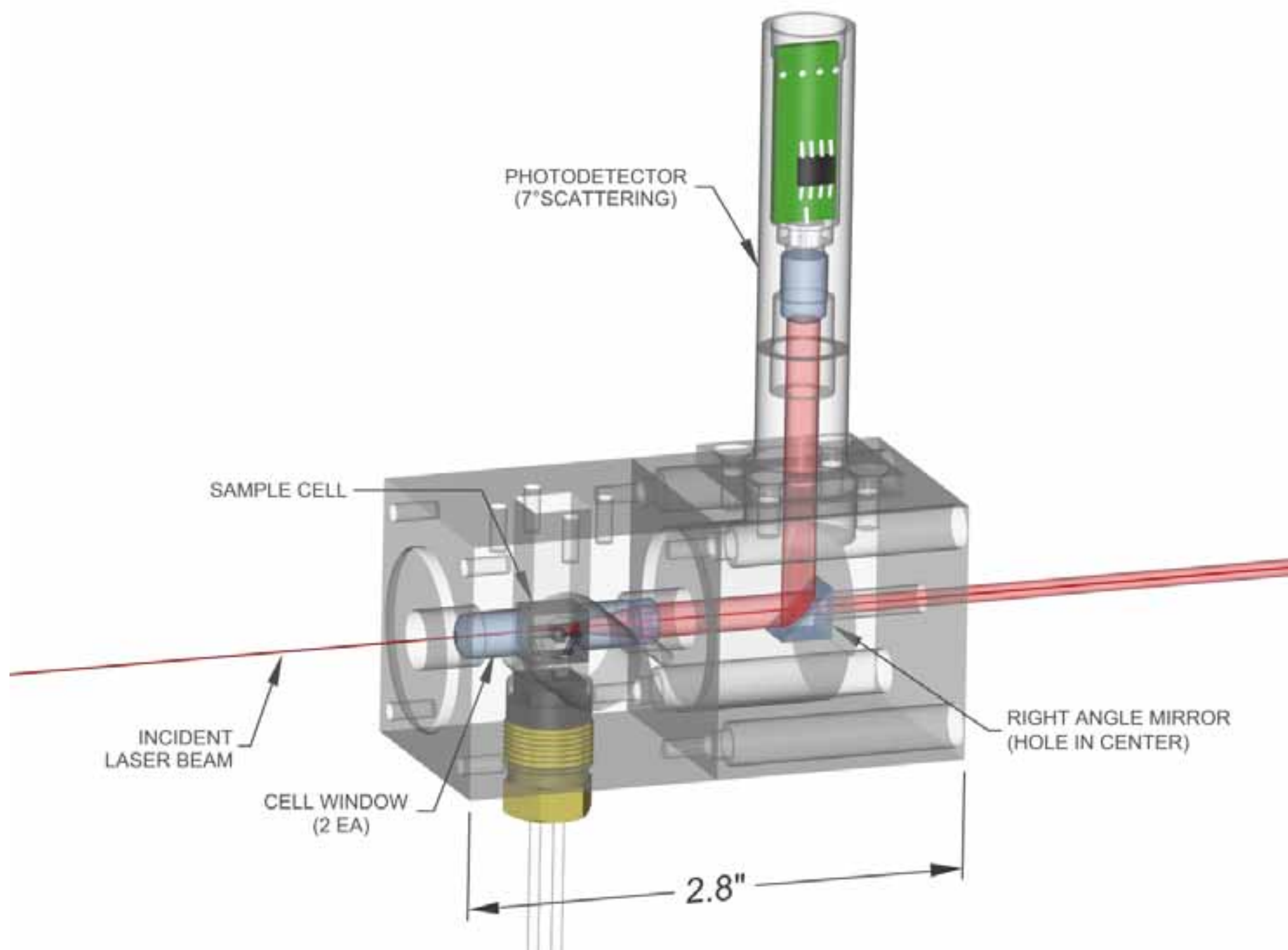


Size of molecule/particle must be significant compared to wavelength of light



Angular Dependence
Angular Scattering Function
1,0500
1,0000
0,9500
0,9000
0,8500
0,8000
0,7500
0,7000
0,6500
0,6000
0,5500
0,5000
0,4500
0,4000
0,3500
0,3000
0,2500
0,2000
0,1500
3
15
50
150
Radius [nm]
7 deg
15 deg
30 deg
45 deg
60 deg
90 deg
$P(\theta)=2\left[\frac{e^{-x}-(1-x)}{x^2}\right]$
with
$x=\frac{8}{3}\left[Rg\frac{\pi n}{\lambda}\sin(\theta/2)\right]^2$

Low Angle Light Scattering - LALS



Relationship between Size and Molecular Weight

Proteins

	Mol. Weight (D)	R_h (nm)
Ribonuclease	13,700	1.93
b-Lactoglobulin	35,000	2.67
Serum albumin	65,000	3.38
Hemoglobin	68,000	3.40
IgG	150,000	5,02
Catalase	250,000	5.38
Thyroglobulin	660,000	7.50

RALS

LALS

IgM	900,000	11.7
DNA	3 Mio	70

Polysaccharides/PEG

MW	R_h (nm)
12,000	2.62
50,000	5.62
80,000	7.27
150,000	9.81

RALS

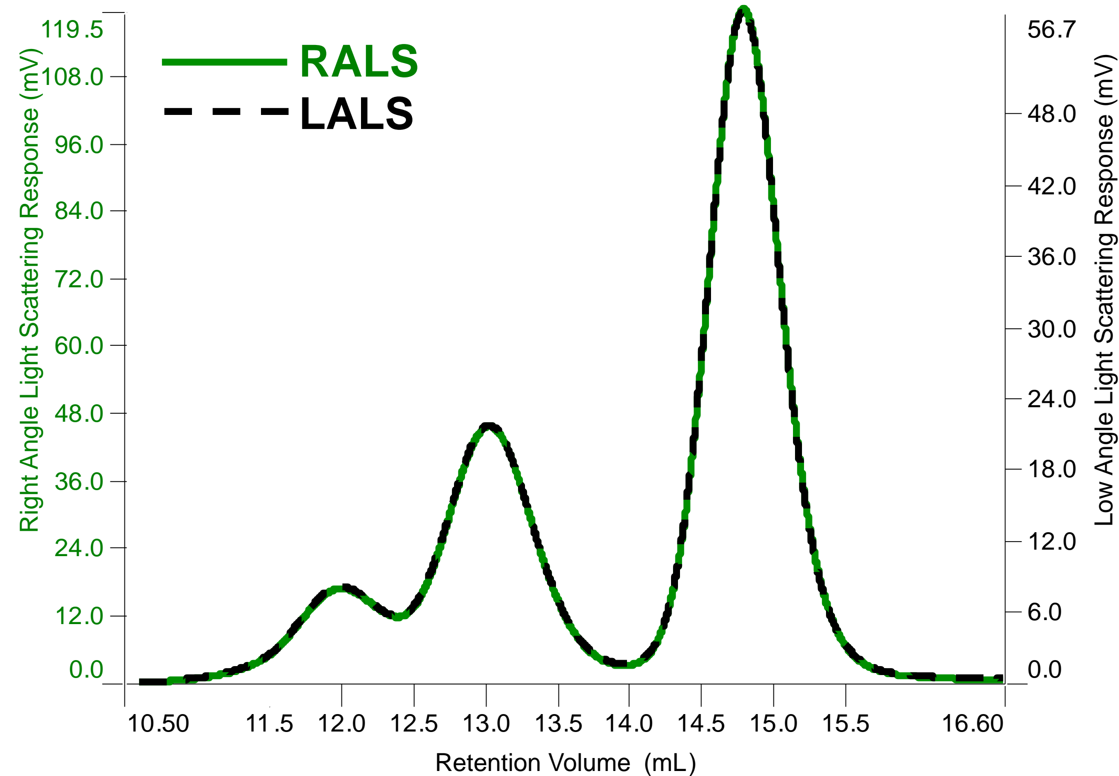
LALS

270,000	12.74
410,000	15.07
670,000	18.45

Normalised LS signals show no angular dependence for proteins

– Molecular weight requires only RALS

– Can not measure size by light scattering alone



Addition of a Viscosity Detector gives hydrodynamic radius

Intrinsic Viscosity

Intrinsic viscosity is the concentration normalized viscosity of the sample in solution at infinite dilution.

$$IV = [\eta] = \left.\frac{\eta_{sp}}{c}\right|_{c \to 0}$$

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

η_{sp} is called the specific viscosity of the solution whose concentration is C.

η_o is the Solvent Viscosity.

η is the Solution Viscosity.

In GPC, concentrations of solutions are sufficiently dilute that the extrapolation to zero concentration is negligible.

Hydrodynamic Radius

$$[\eta] = \frac{2.5}{\rho} = \frac{2.5 \cdot V_h}{M}$$

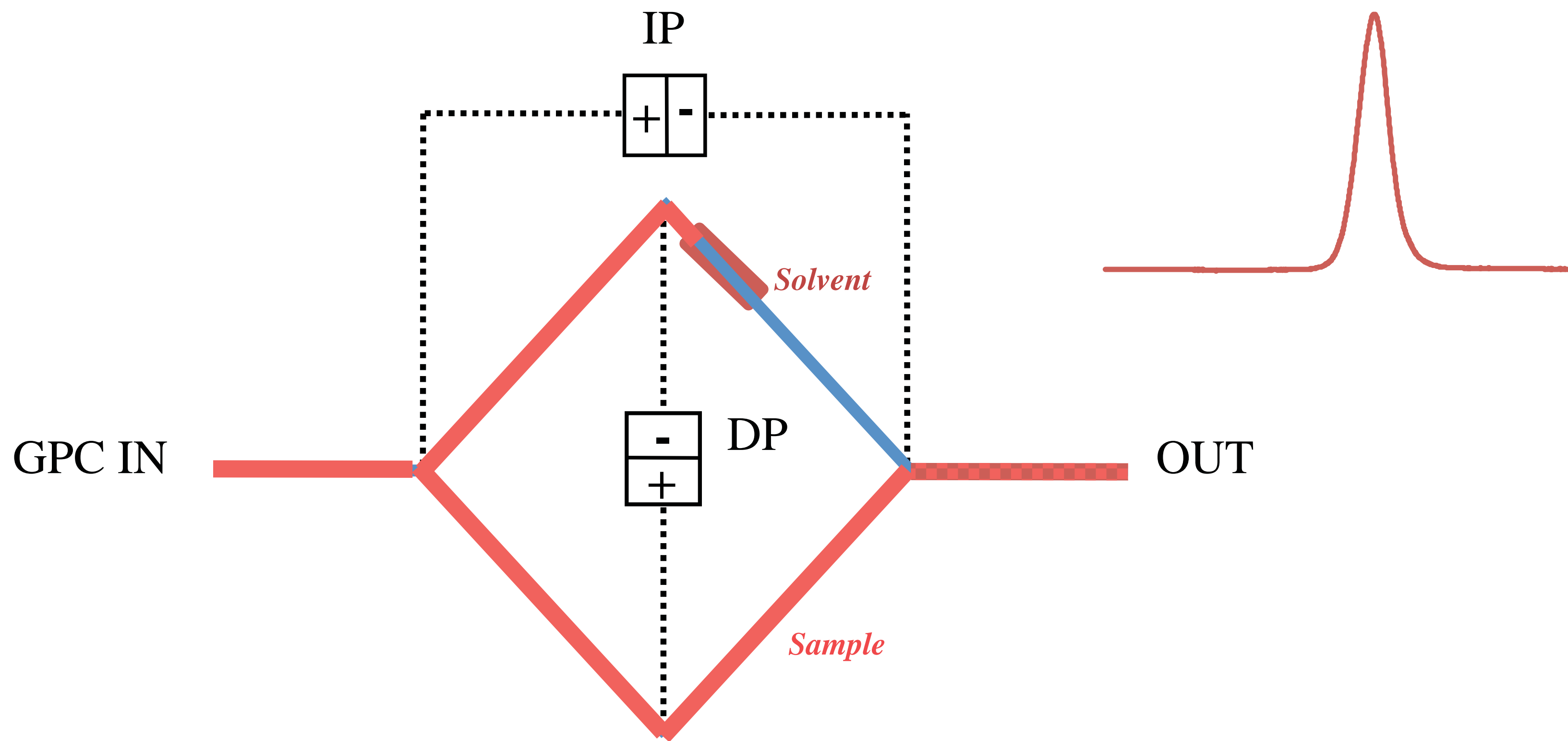
$$[\eta]\ M = 2.5 \cdot V_h = 2.5 \cdot \left(4/3\,\pi\, R_h^3\right) = 10.47\ R_h^3$$

Intrinsic Viscosity is inverse proportional to the density

Intrinsic Viscosity x Molecular weight is proportional to the hydrodynamic radius of a sample

Intrinsic Viscosity: from Viscosity Detector
Molecular Weight: from Light Scattering Detector

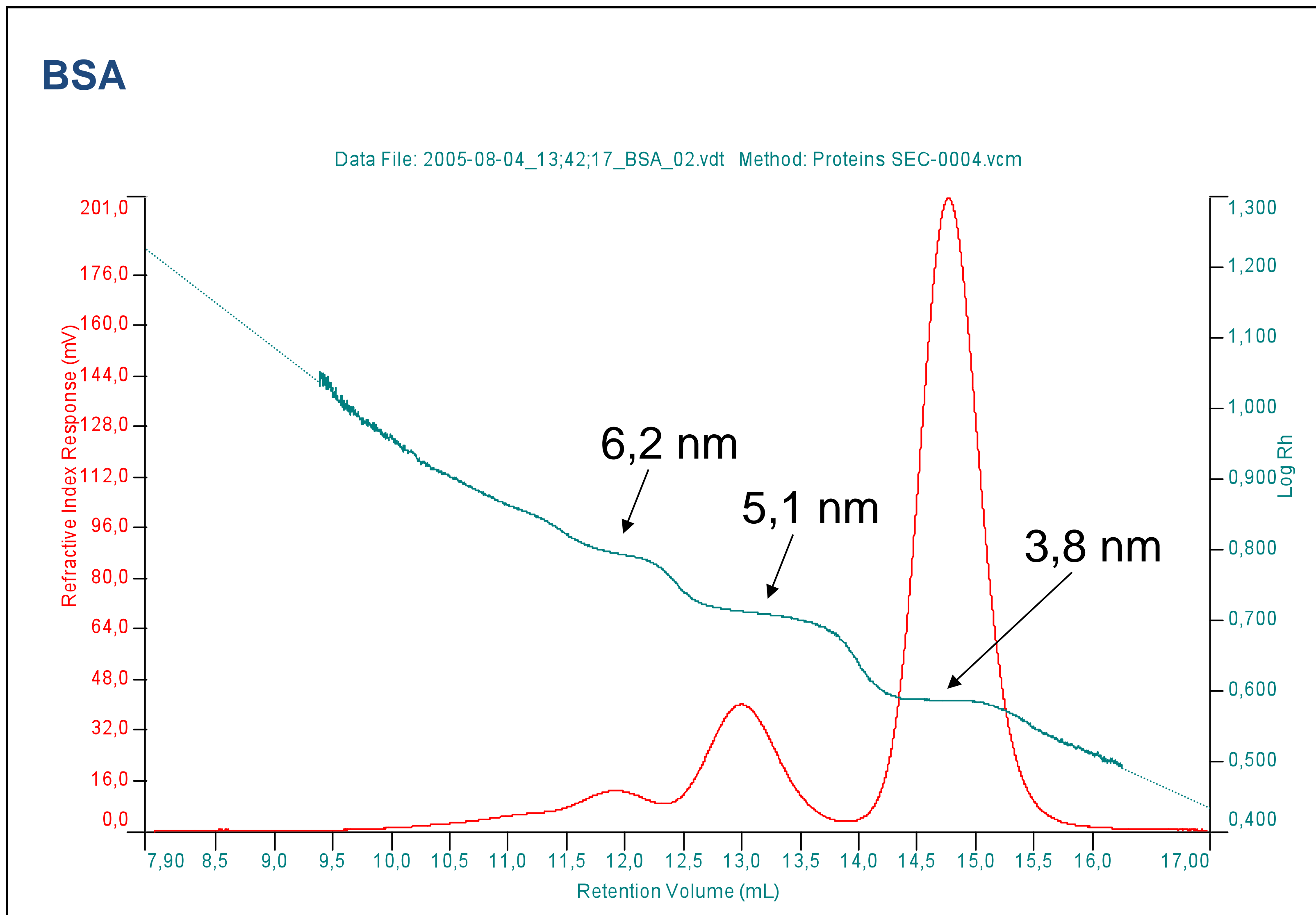
Capillary Differential Viscosity Detector



Wheatstone bridge concept
M. Haney, 1983

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

Hydrodynamic Radius



Online Quality control

- UV
- Refractive index
- Viscosity
- Low angle light scattering

Viscotek/Malvern

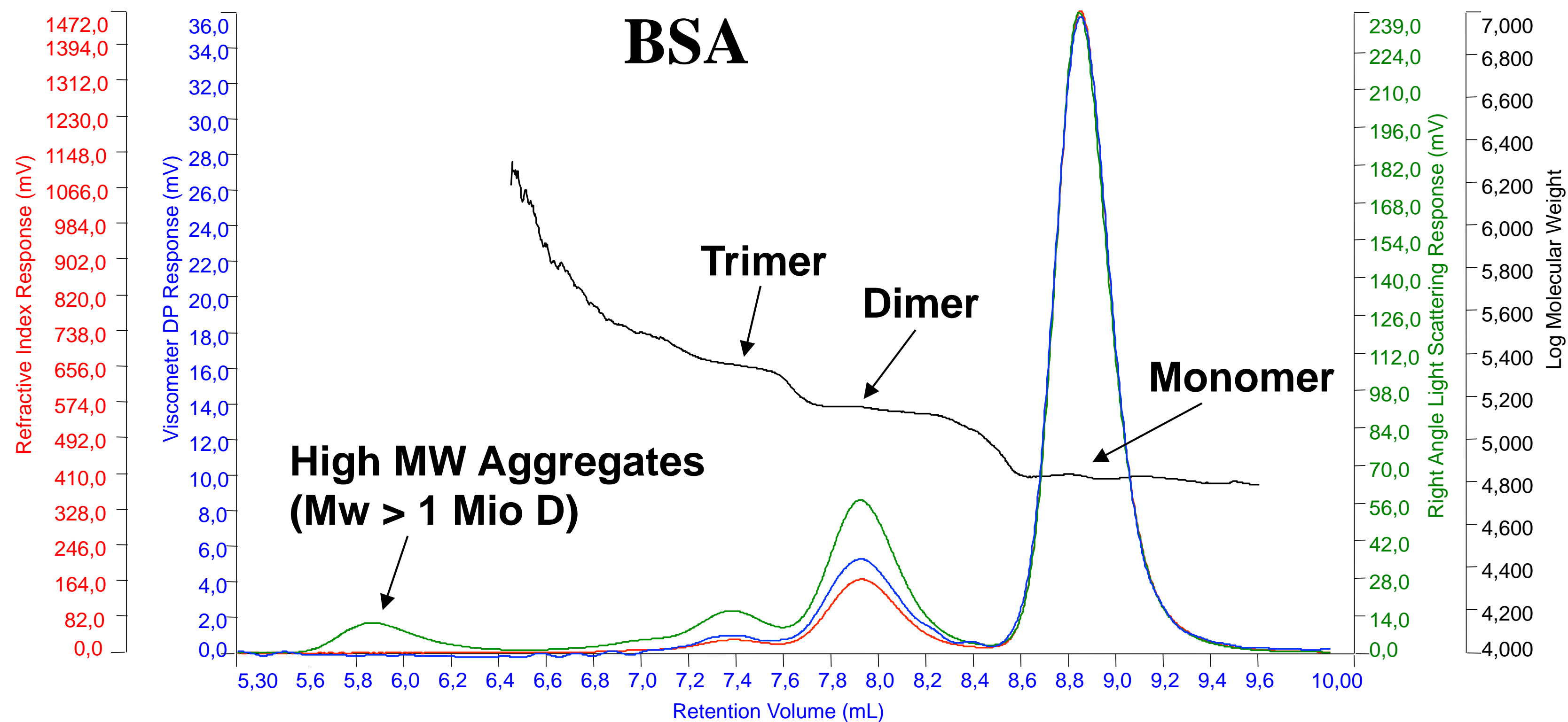
What are the detectors responding to?

Refractometer $= K_{RI} \times dn/dc \times Conc$

UV-Detector $= K_{UV} \times dA/dc \times Conc$

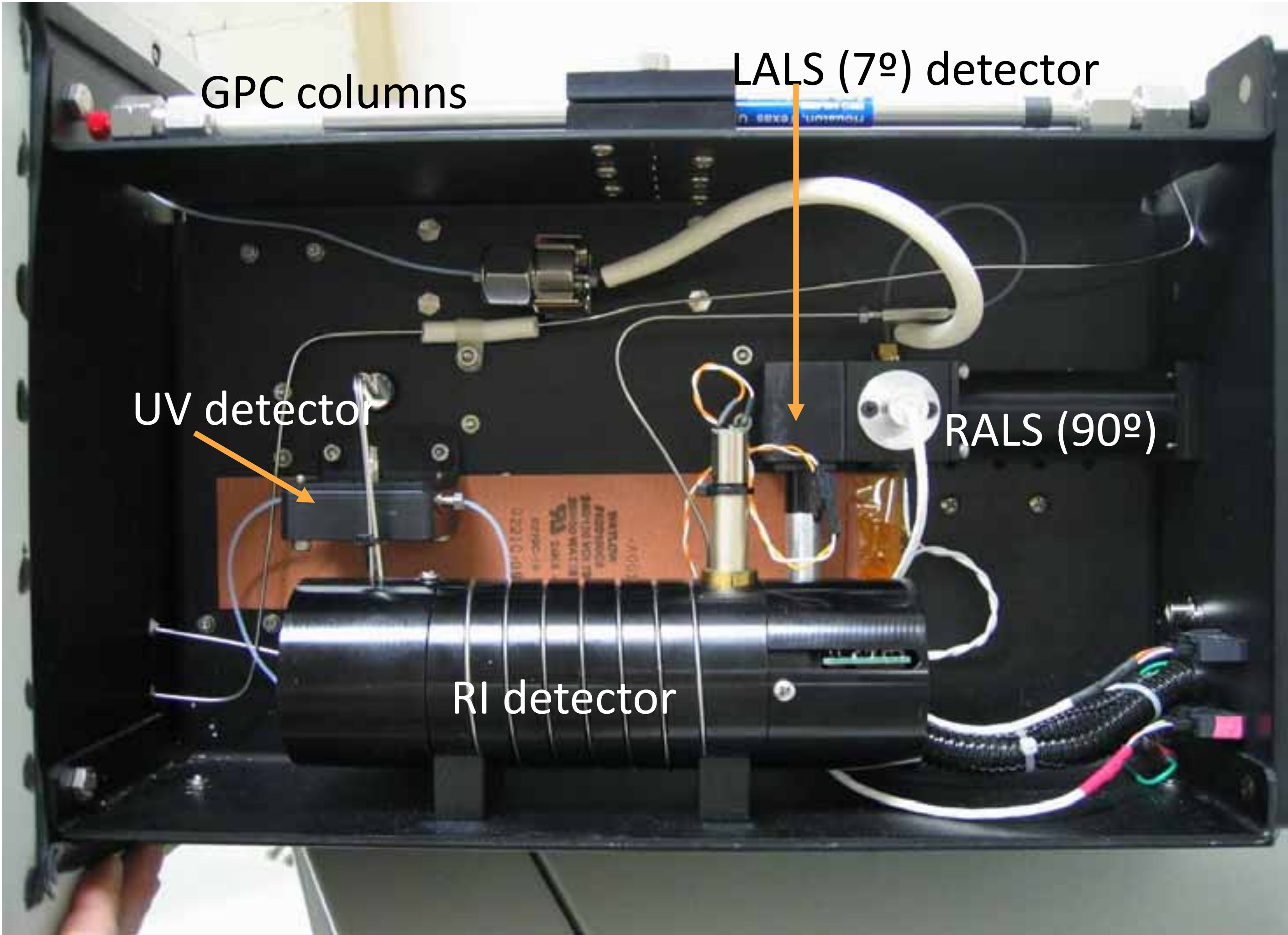
Viscometer $= K_{Visc} \times \text{Intrinsic Viscosity} \times Conc$

Light Scattering $= K_{LS} \times Mw \times (dn/dc)^2 \times Conc$

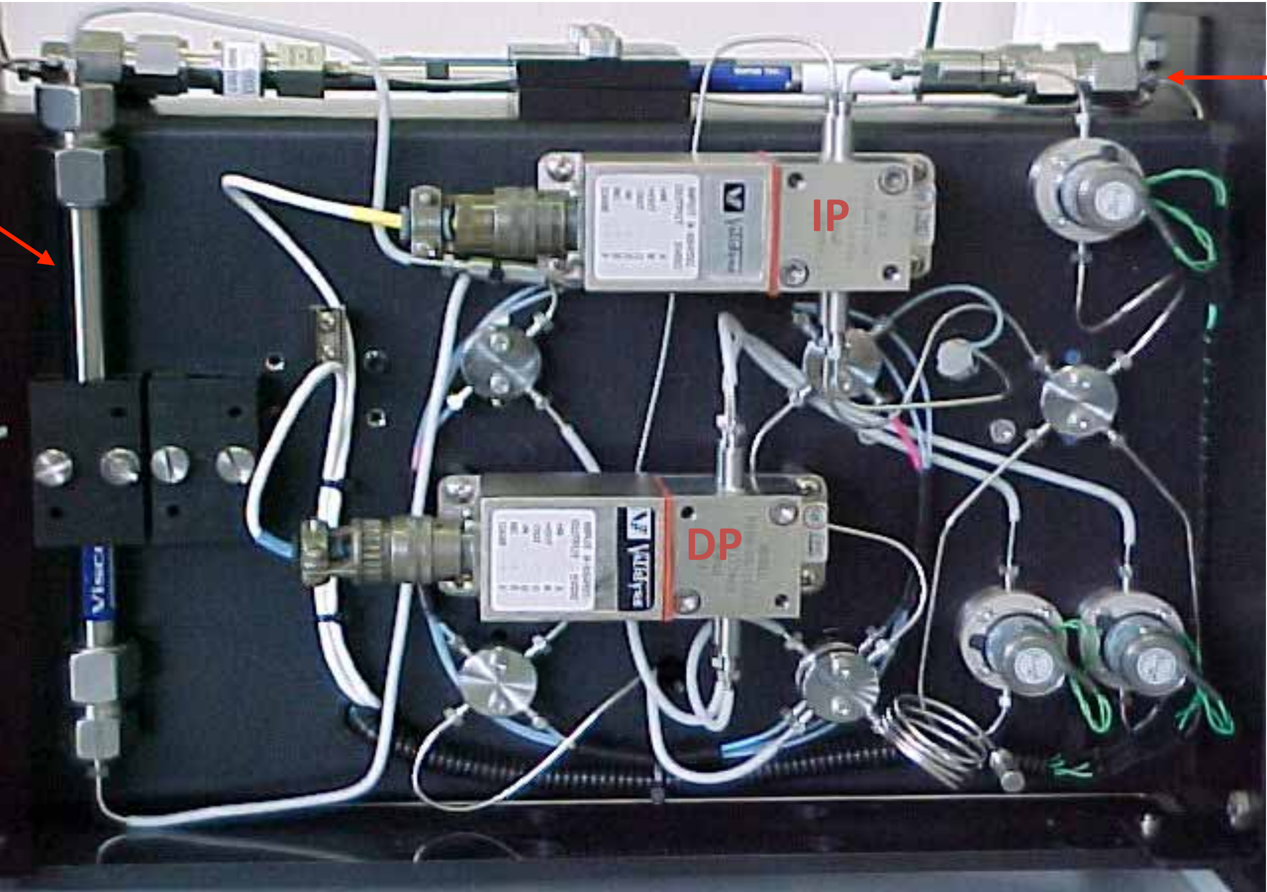


	Mw (D)	IVw (dl/g)	Rh (nm)	Weight Fraction (%)
Monomer	66.430	0,056	3,88	87
Dimer	133.000	0,071	5,32	11
Trimer	201.000	0,095	6,69	1,5
	Light Scattering	Viscometer		Refractive Index

Laser Light Scattering Detector, Refractive Index Detector and UV-Cell



Balanced Bridge 4-Capillary Viscometer Detector



Online purification & QC

Sample optimization

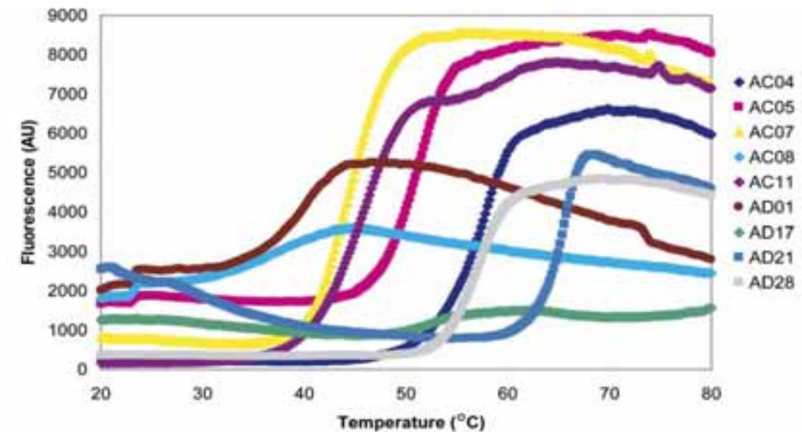
- Reduced Gel problematic: change purification protocol
- NR Gel problematic: check cysteines
- Protein aggregation, folding stability:
  - Size exclusion, light scattering, CD, NMR, thermofluor

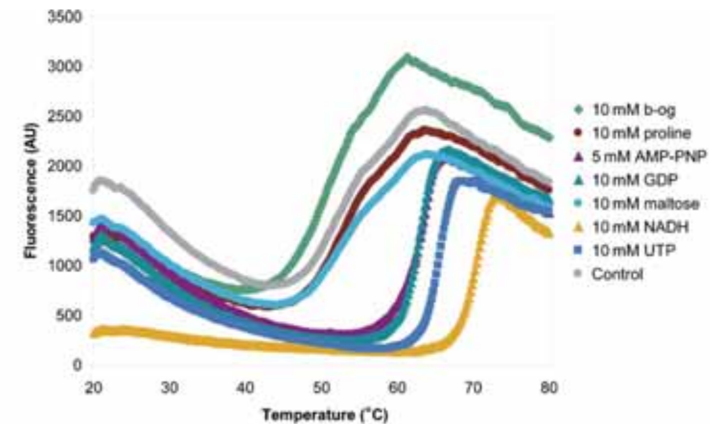
Thermal stability

- Thermofluor
- Modified real-time PCR machine
  - Add hydrophobic fluorescent probe
  - When protein unfolds…
  - Fluorescence increases

Thermal stability

- Check protein stability
- Additive/ligand screen





Ericsson *et al Analytical Biochemistry* **357** (2006) 289

Sample optimization

- Reduced Gel problematic: change purification protocol
- NR Gel problematic: check cysteines
- Protein aggregation, folding stability:
  - Size exclusion, light scattering, CD, NMR, thermofluor
- Modify buffers, additives
- If nothing works: change construct

Additives?

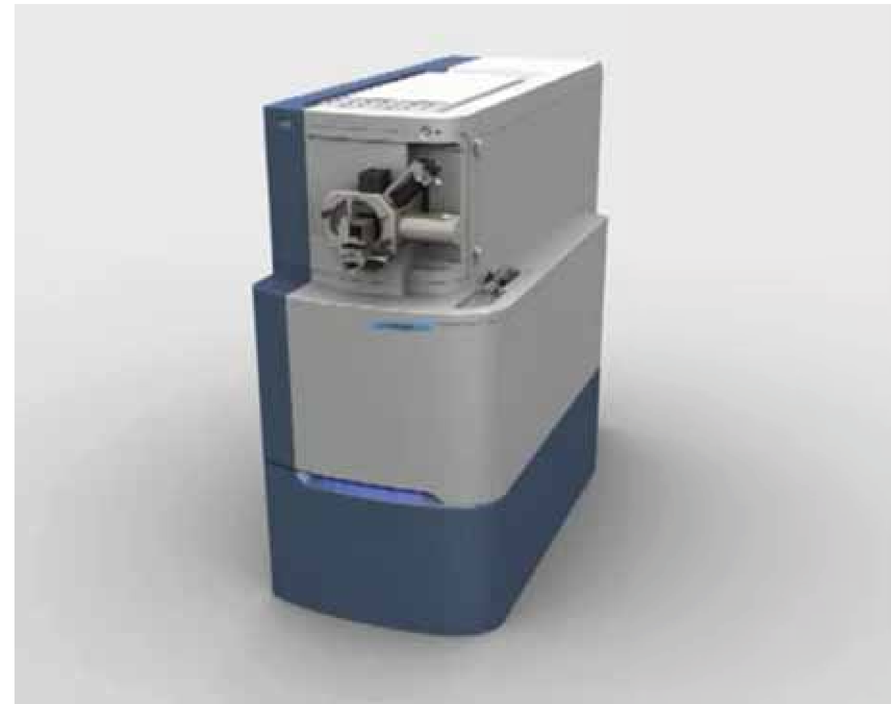
- **DTT**
- **Glycerol**
- **Detergents at less than 2xCMC**

  0.1% 1-s-Nonyl-β-D-thioglucoside
  0.2% n-Decanoylsucrose
  0.3% n-Nonyl-β-D-maltoside
  0.4% DDAO
  0.5% C8E5
  0.8% FOS-Choline®-10
  1.1% FOS-Choline®-9

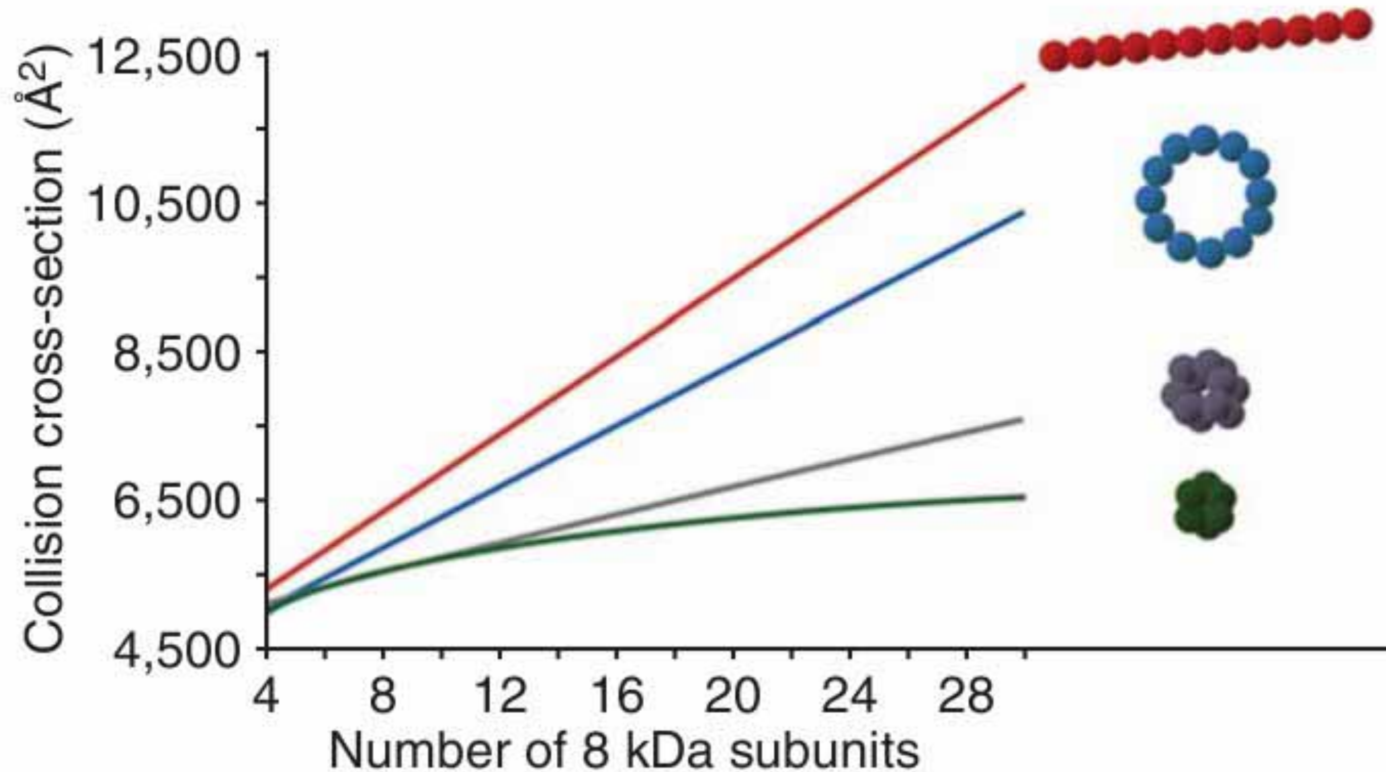
Mass spectrometry

- Traditional MS:
  - Confirm mass
  - Confirm sequence
  - Check folding state

- MS/MS + Ion mobility :
  - Detailed folding state
  - Protein-protein interactions
  - Whole protein size…



Ion mobility derived particle size



Ruotolo et al *Nature Protocols* (2008) **3**, 1139

in combination with SAXS…

Conclusions

- QC at home is crucial
- But soon we hope to do it for you at EMBL@PETRA3
- Some quality control methods can provide useful complementary data
- This all can filter the garbage “in” bit

But the garbage out?

Acknowledgements

- Malvern Instruments

  Bernd Tartsch