BioProcess International™

Analytical and Quality Summit

Protein Characterization by Static Light Scattering

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Yale University

Workshop:
“Oligomerization and High Molecular Weight Species Determination“

- Light Scattering Technologies
  - Static and dynamic light scattering
  - Parameters derived from SLS and DLS measurements
- Detection and differentiation between low order oligomers and high molecular weight aggregates
- Flow Mode Light Scattering Applications
  - Molar mass distributions and differences in populations
  - Morphology of aggregates from light scattering measurements (static and dynamic)
  - Determination of dimerization constant from SEC-LS measurements

Light Scattering Experiments

- Static (classical)

  time-averaged intensity of scattered light

- Dynamic (quasielastic)

  fluctuation of intensity of scattered light with time

Measurements:

- batch mode
- “in-line” mode combined with a fractionation step,

  i.e. chromatography, mainly Size Exclusion Chromatography, Flow Field Fractionation

Light Scattering Experiments

- **Static (classical)**

time-averaged intensity of scattered light

- **Dynamic (quasielastic)**

fluctuation of intensity of scattered light with time

Parameters derived:

- Molar Mass (weight-average) accuracy ~5%
- ($<r_g^2>^{1/2}$) root mean square radii

  for ($<r_g^2>^{1/2}$)> (λ/ 20) ~ 15 nm
- A_2 second virial coefficient

Parameters derived:

- D_T translation diffusion coefficient
- R_h hydrodynamic radius (Stokes radius)

Uncertainty of ~10% for monodisperse sample

Rayleigh-Debye-Zimm formalism

$$\frac{K^*c}{R(\theta)} = \frac{1}{M_w P(\theta)} + 2A_2 c$$

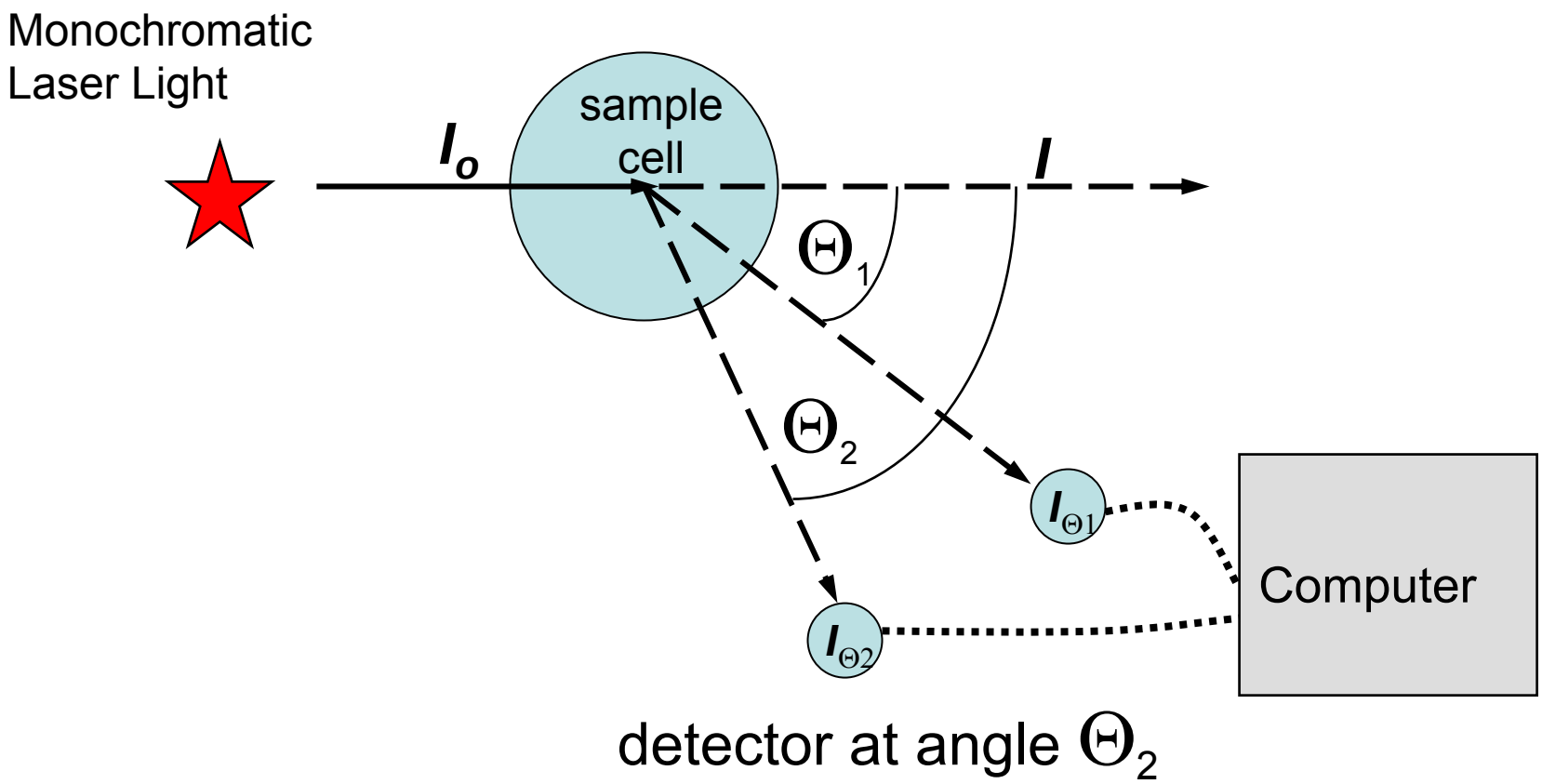
	R(Θ)	Rayyleigh ratio (excess scattered light)
	c	sample concentration (g/ml)
	Mw	weight-average molecular weight (molar mass)
A_2		second virial coefficient (ml-mol/g2)
P(Θ)		form factor (angular dependence)
K		optical constant [4π2n2 (dn/dc)2 /(λo4NA)]

Stokes-Einstein

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

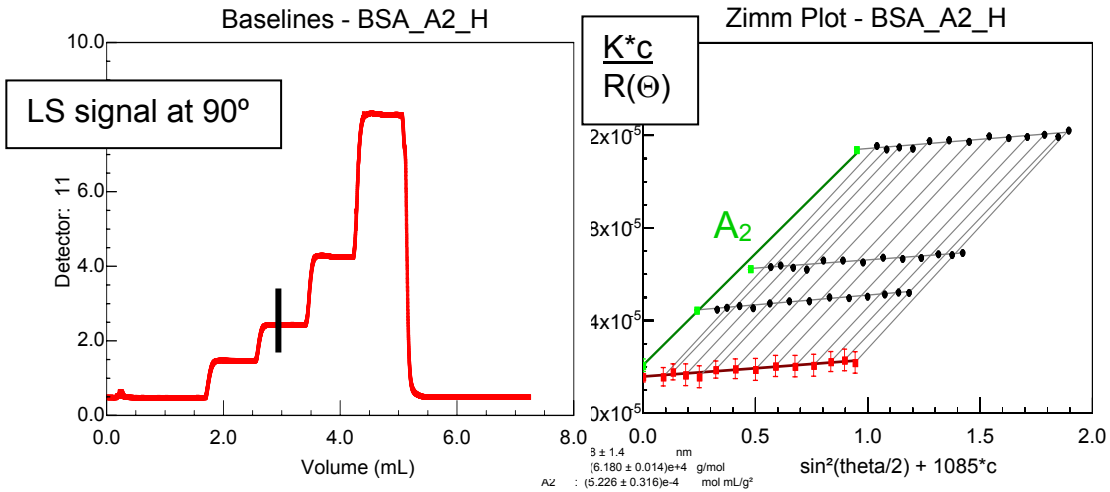
R_h		hydrodynamic radius
η		solvent viscosity
	D_T	translational diffusion coefficient
	k	Boltzmann constant
	T	temperature

Light Scattering Experiments



Determination of Molar Mass and second virial coefficient from a batch static LS experiment

BSA 66 kDa



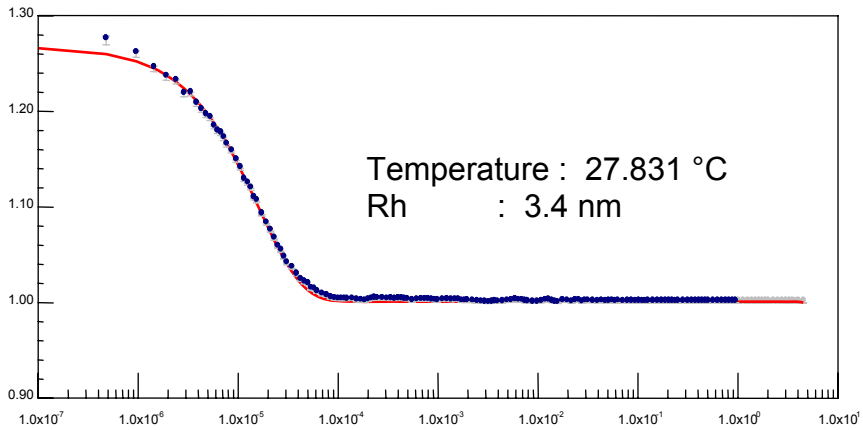
$$\frac{K^*c}{R(\theta)} = \frac{1}{MwP(\theta)} + 2A_2c$$

and Rh from DLS

Zimm plot analysis of static light scattering data

Mw = 62 kDa

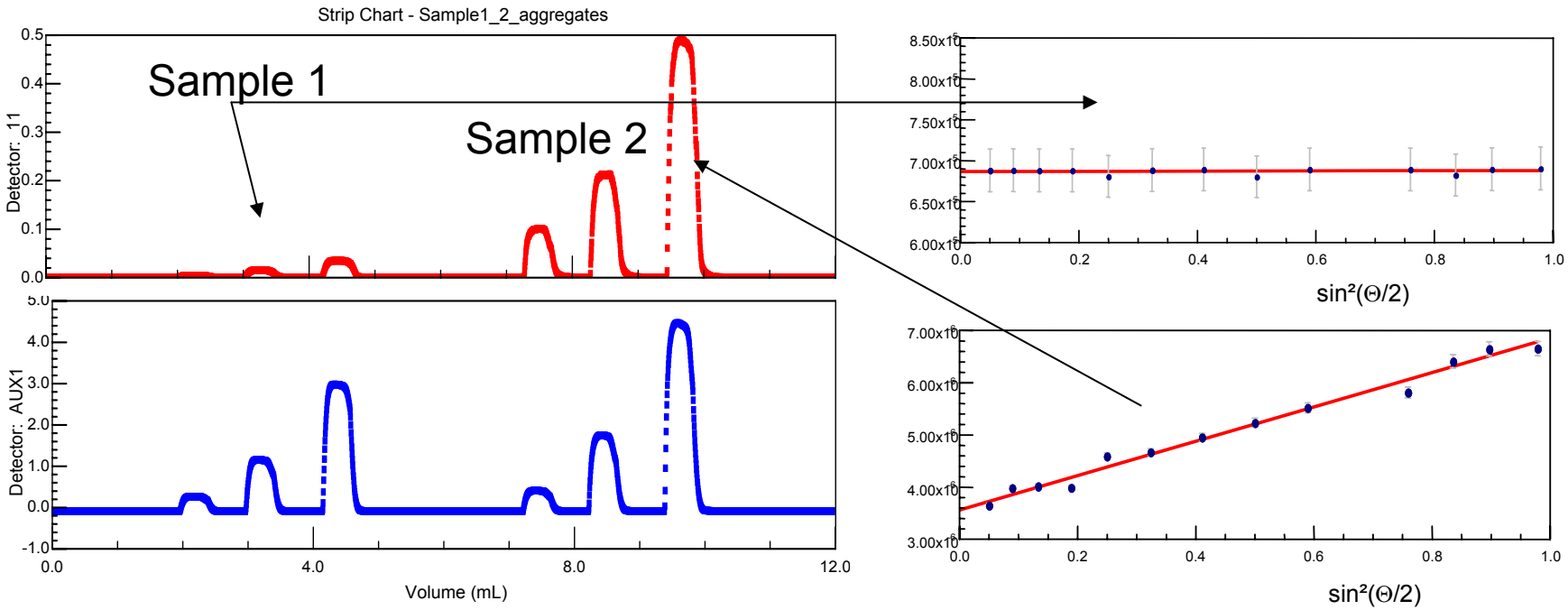
A_2 = (5.226 ± 0.316)e^{-4} mol mL/g²



B. H. Zimm, (1948) J. Chem. Phys., 16, 1099

Batch Mode Static MALLS experiment

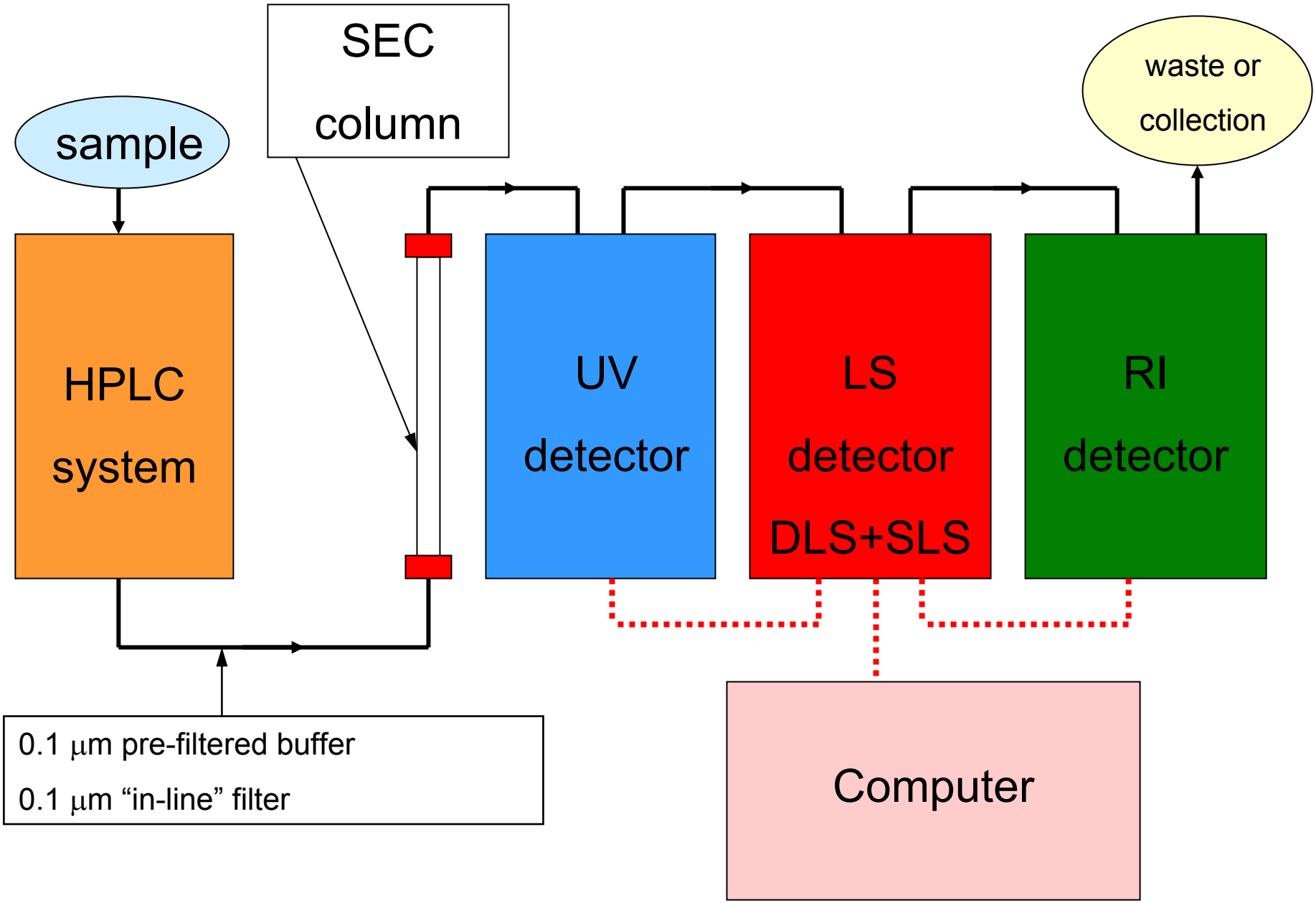
Monomer 14 kDa



Sample	Weight Average MM, Mw ± SD* [kDa]	RMS [nm]
1	15 ± 1	0
2	126 ± 8	56 ± 10

Angular dependence of scattered light clearly indicates presence of aggregates

Missing information: how much and what size?


SEC
column
waste or
collection
sample
HPLC
system
UV
detector
LS
detector
DLS+SLS
RI
detector
0.1 μm pre-filtered buffer
0.1 μm “in-line” filter
Computer

Ovalbumin 43 kDa

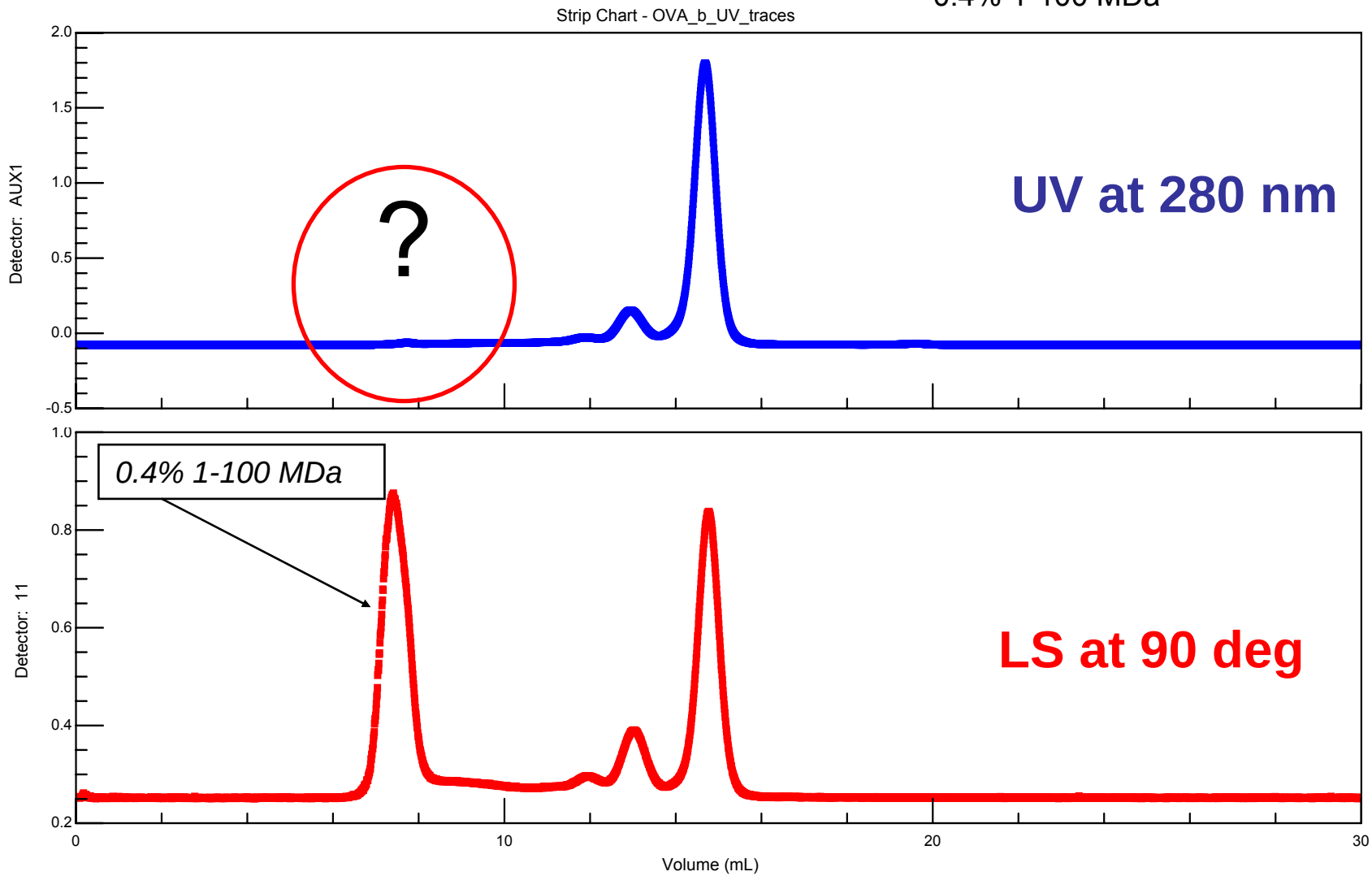
88% monomer

8% dimer

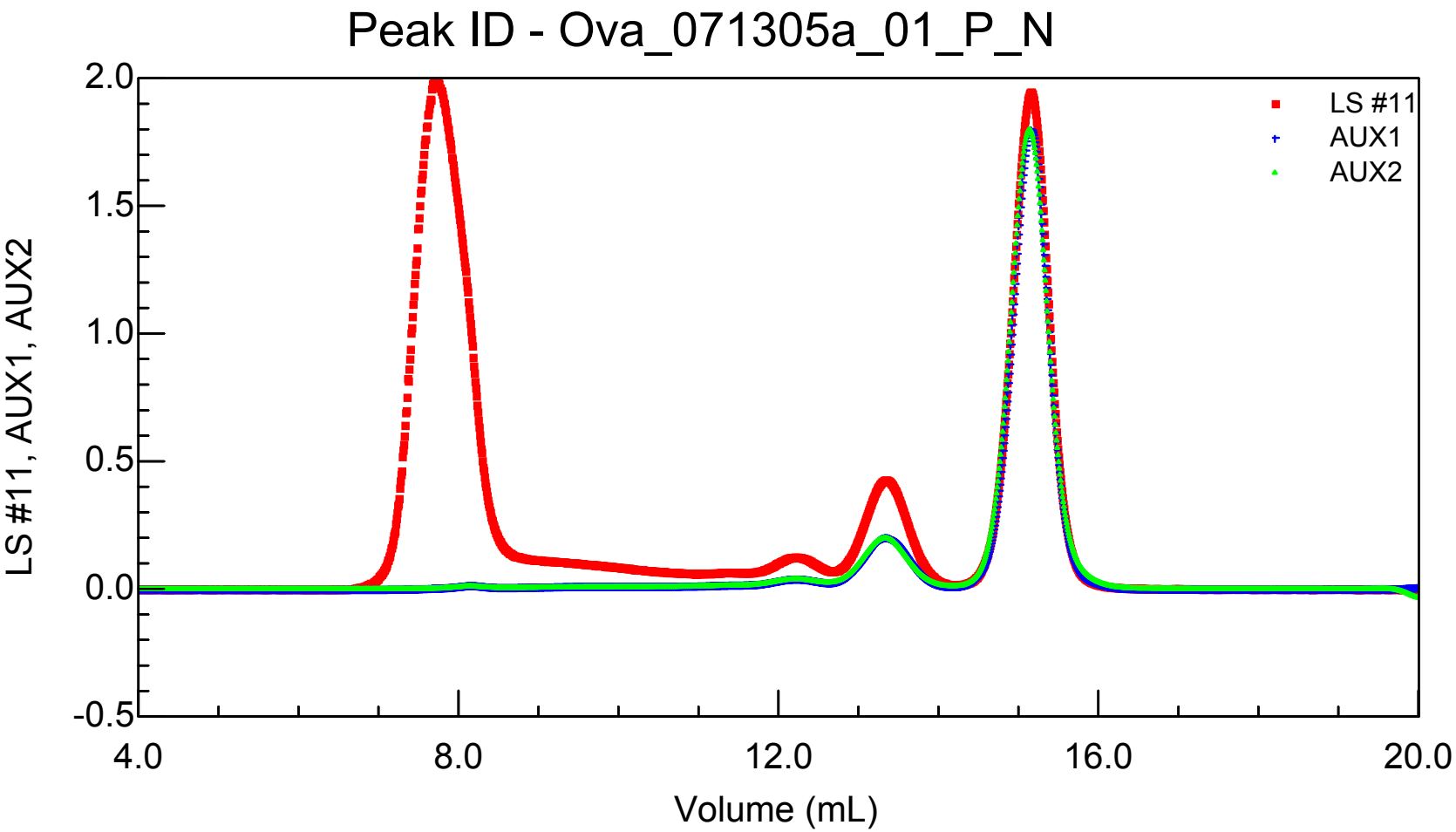
1.5% trimer

3% aggregates < 1MDa

0.4% 1-100 MDa



Three Detector monitoring



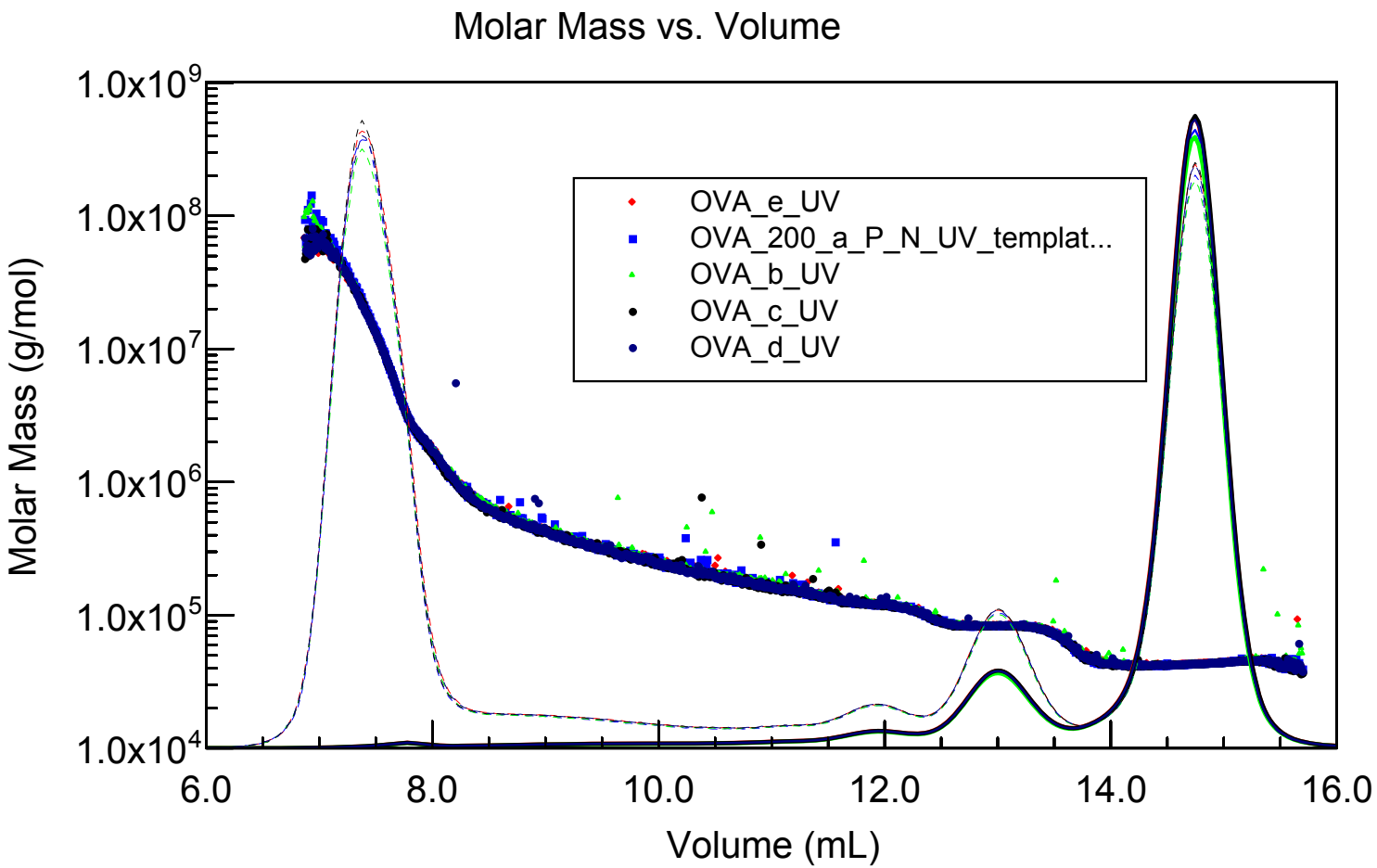
UV at 280 nm

RI

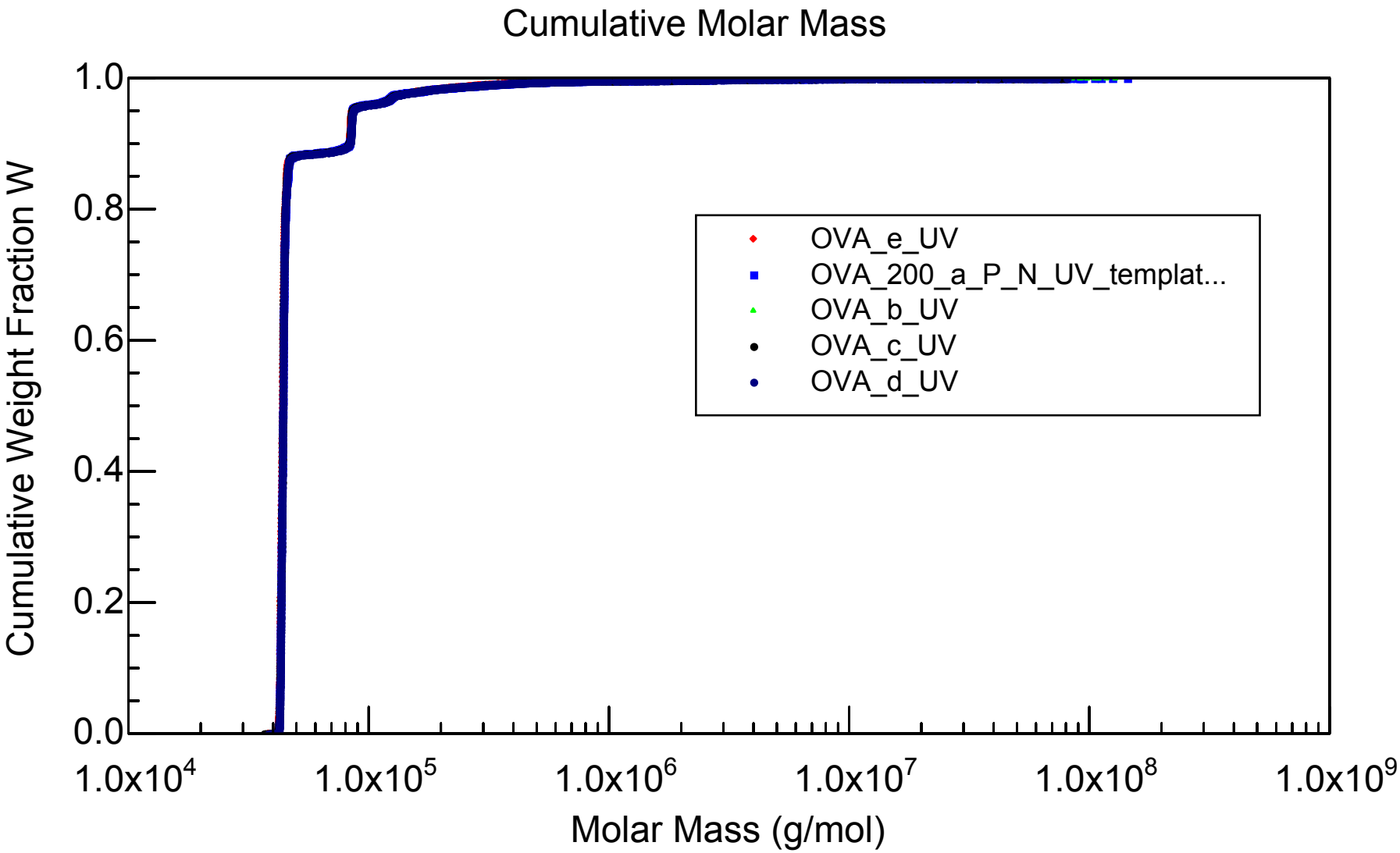
LS at 90°

Molar mass distribution for multiple analyses

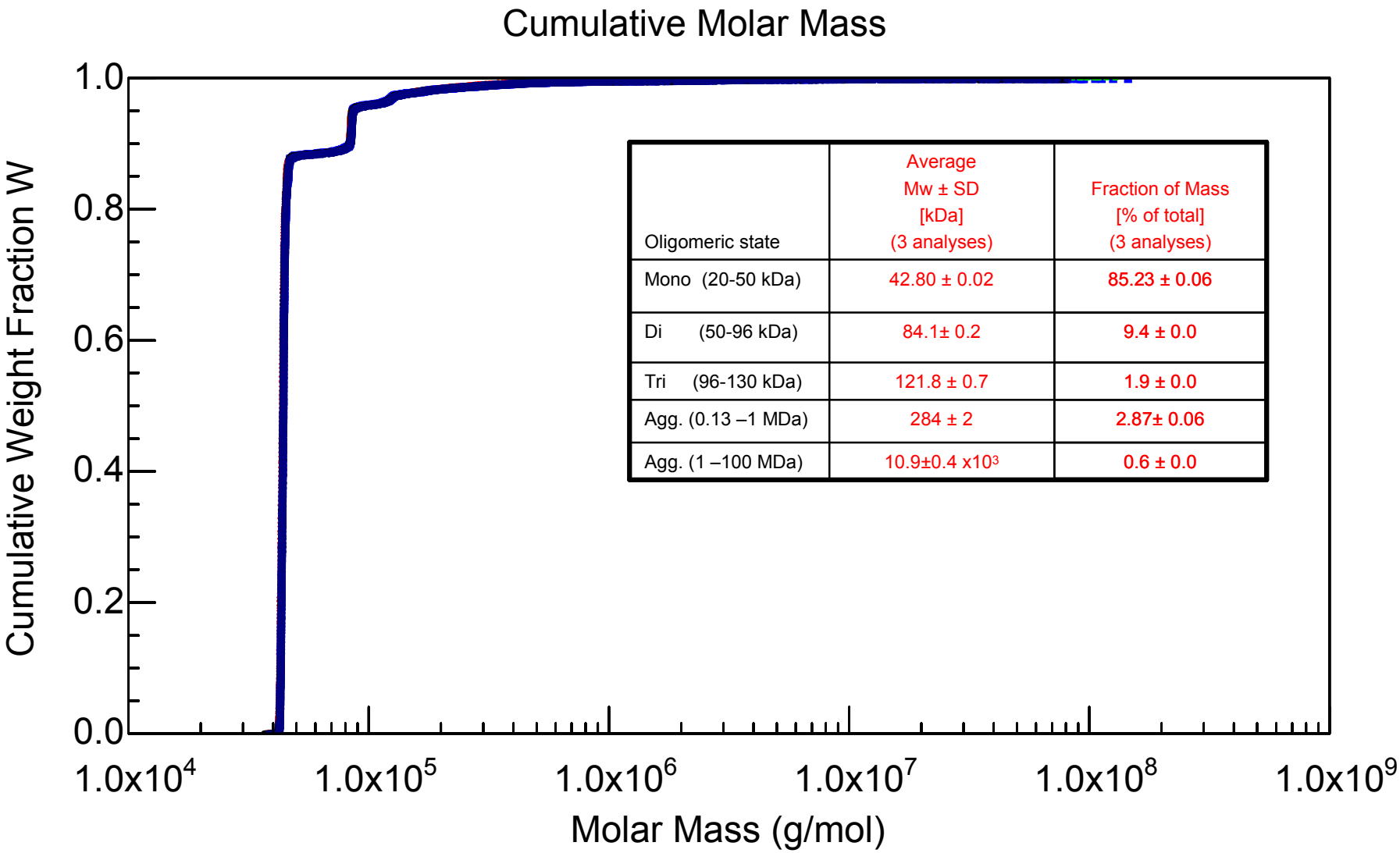
Ovalbumin 43 kDa automated template processing of five data sets



Determination of Weight Fractions

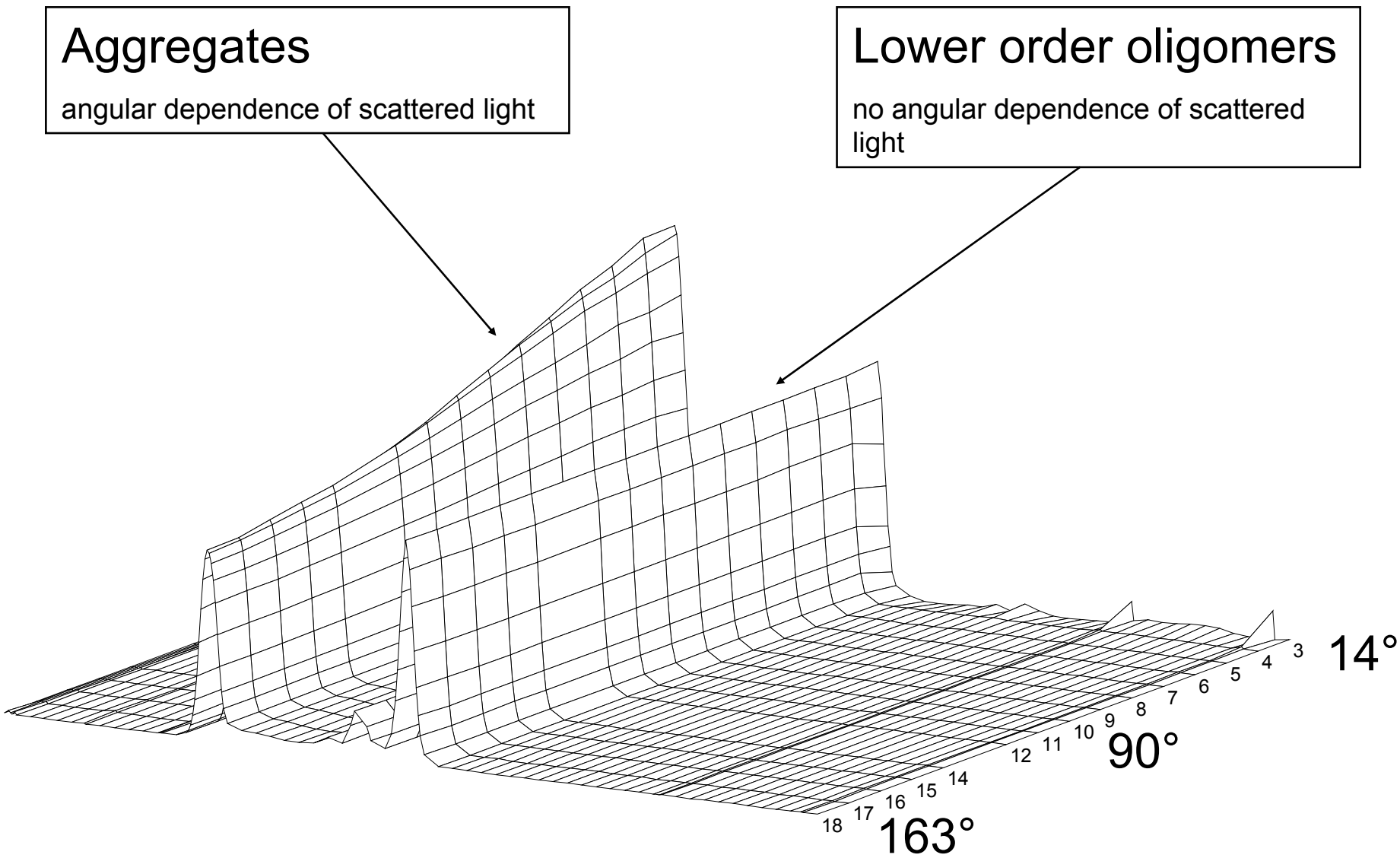


Determination of Weight Fractions



Oligomeric state	Average Mw ± SD [kDa] (3 analyses)	Fraction of Mass [% of total] (3 analyses)
Mono (20-50 kDa)	42.80 ± 0.02	85.23 ± 0.06
Di (50-96 kDa)	84.1± 0.2	9.4 ± 0.0
Tri (96-130 kDa)	121.8 ± 0.7	1.9 ± 0.0
Agg. (0.13 –1 MDa)	284 ± 2	2.87± 0.06
Agg. (1 –100 MDa)	10.9±0.4 x10^3	0.6 ± 0.0

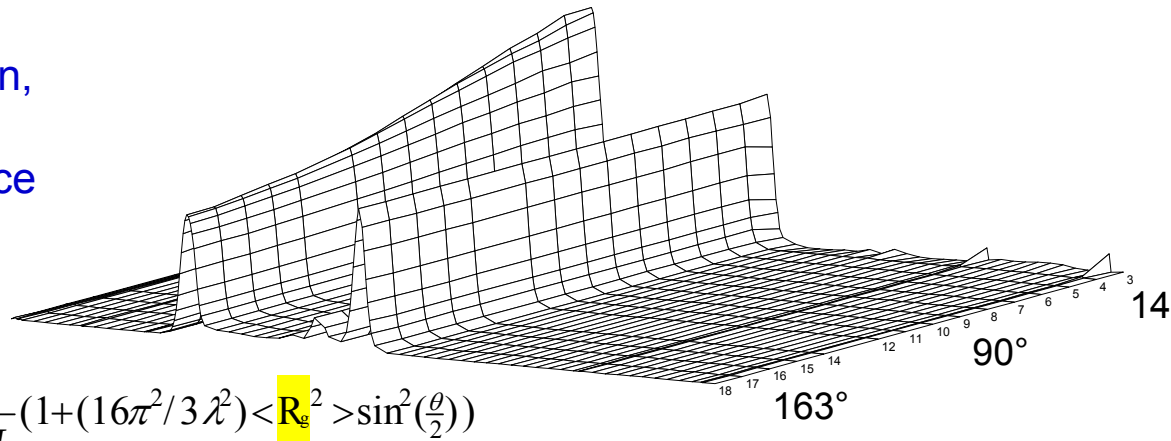
Ovalbumin 43 kDa



Morphology of aggregates from angular dependence of LS signal;

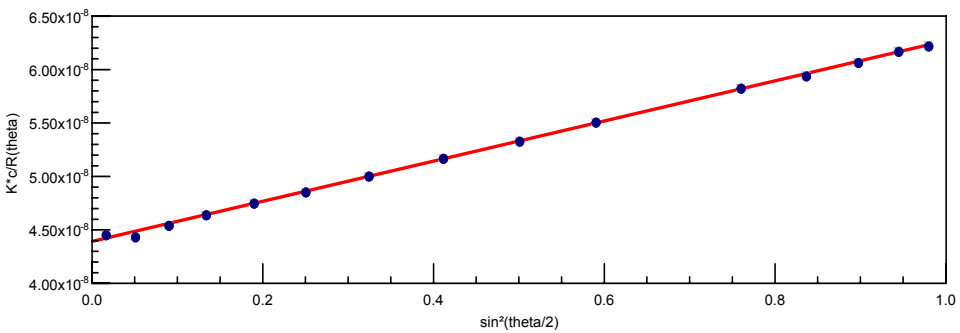
size determination- Rg

Determination of radius of gyration, R_g, (root mean square radius, R.M.S.,) from angular dependence of scattered light



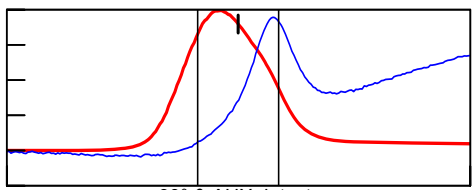
Zimm Plot

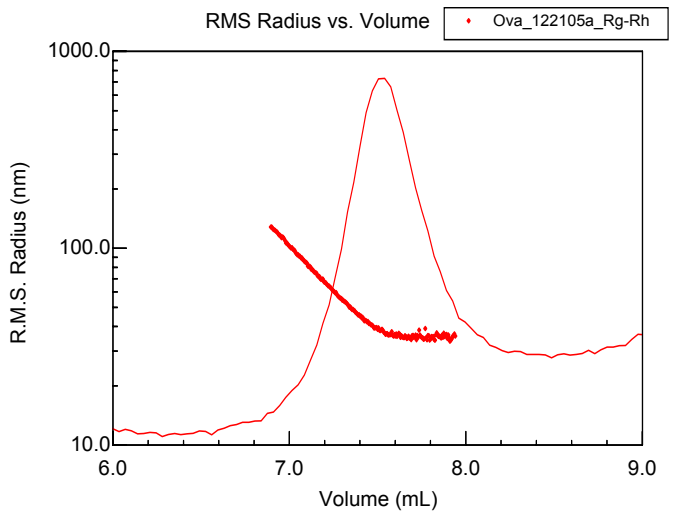
$$\frac{K^*c}{R(\theta)} = \frac{1}{M_w}\left(1+\left(16\pi^2/3\lambda^2\right)\langle R_g^2\rangle \sin^2\left(\frac{\theta}{2}\right)\right)$$



Peak, Slice : 1, 944
Volume : 7.867 mL
Fit degree : 1
Conc. : (1.915 ± 0.020)e-6 g/mL
Mw : (2.277 ± 0.024)e+7 g/mol

Radius: 46.8±0.2 nm





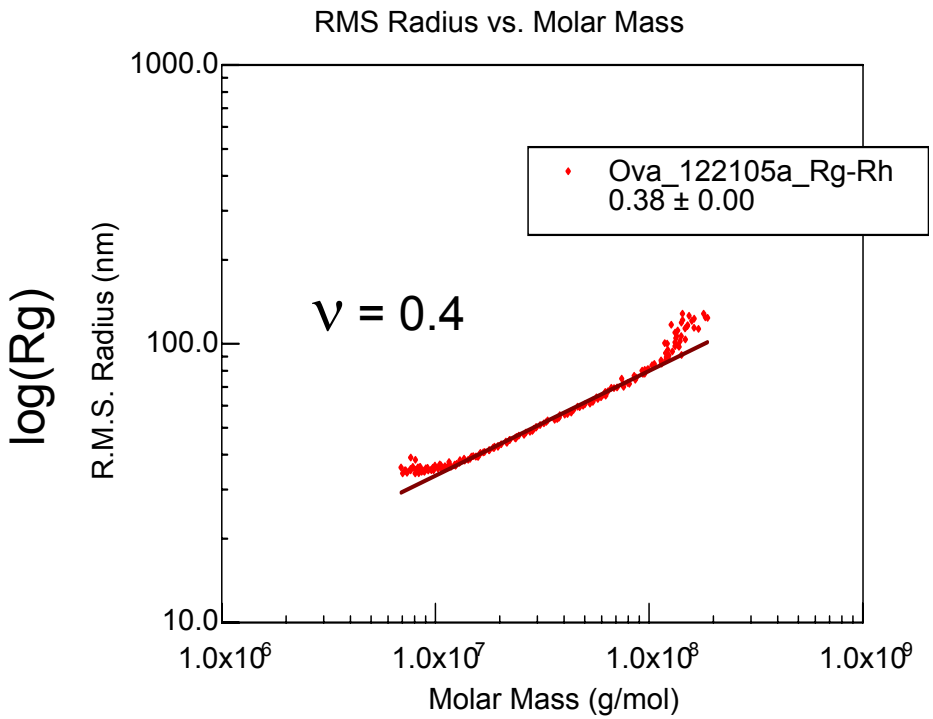
Inferring conformational information from the relationship between molecular size (Rg) and molecular weight (Molar Mass)

$$R_g \sim M^{\nu}$$

$\log(R_g)$ *versus* log(MM)

Slope = ν

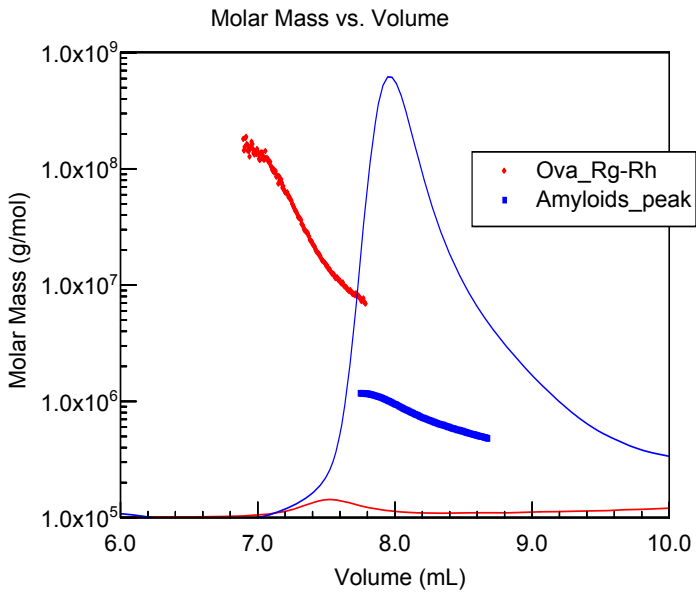
For	ν
Sphere	0.33
Coil	0.5
Rod	1

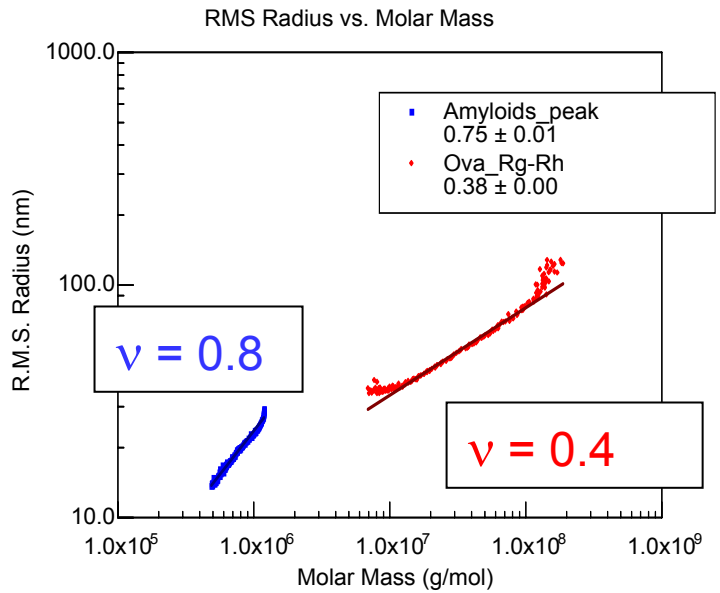


Rollings, J.E. (1992) in *"Laser Light Scattering in Biochemistry"*, Eds. S.E. Harding, D. B. Sattelle and V. A. Bloomfield; p. 275-293

Shape analysis: **log(Rg) *versus* log(MM)**

Aggregates of Ovalbumin *vs.* "amyloid-type" fibers





For	ν
Sphere	0.33
Coil	0.5
Rod	1

Ova_aggr ν = 0.4 Sphere/Coil

Amyloids ν = 0.8 Coil/Rod

Shape analysis: **shape factor $\rho = R_g/R_h$**

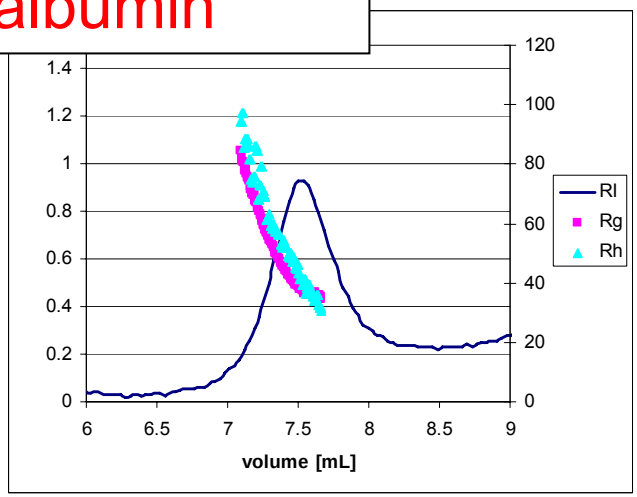
Aggregates of Ovalbumin *vs.* amyloid fibers

Shape factor: $\rho = R_g/R_h$

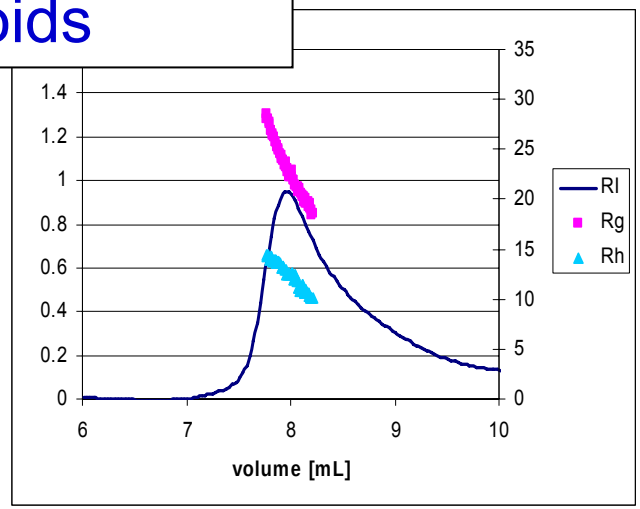
Combination of MALS (R_g) and DLS (R_h)

For	$\rho = R_g/R_h$
Sphere	0.774
Coil	0.816
Rod	1.732

Ovalbumin



Amyloids



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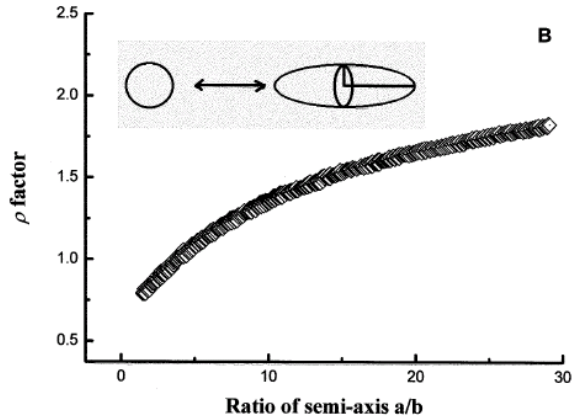
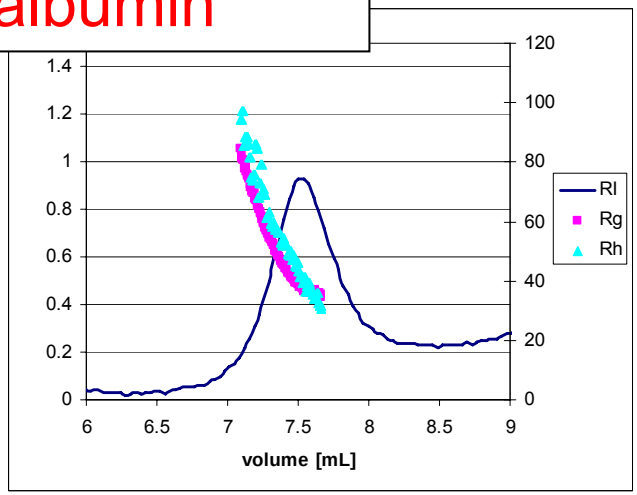
Aggregates of Ovalbumin *vs.* amyloid fibers

Shape factor: $\rho = R_g/R_h$

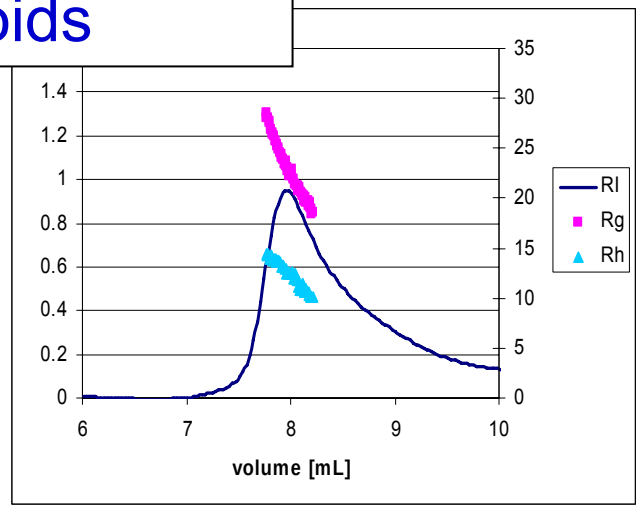


Combination of MALS (R_g) and DLS (R_h)

Ovalbumin



Amyloids

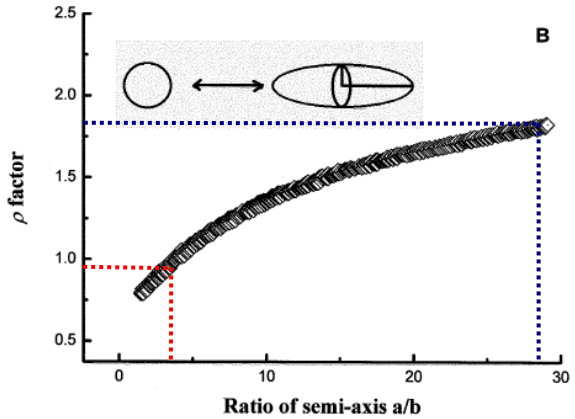


[B] Rizos, A. K., Spandidos, D. A., and Krambovitis, E., (2003) *Int. J. Med.,* 12, 559-563

Shape analysis: **shape factor $\rho = R_g/R_h$**

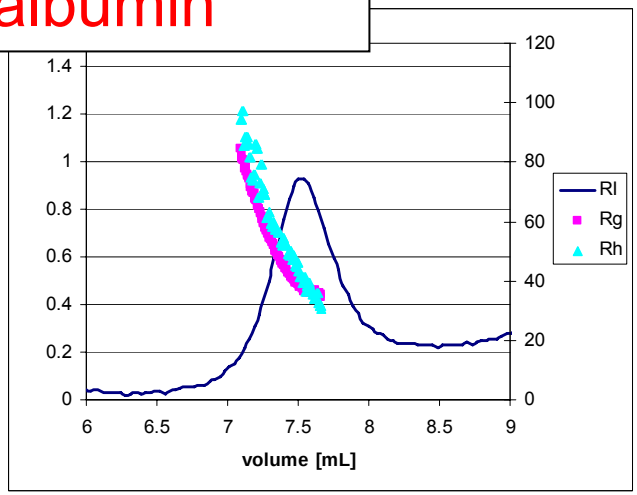
Aggregates of Ovalbumin *vs.* amyloid fibers

Shape factor: $\rho = R_g/R_h$



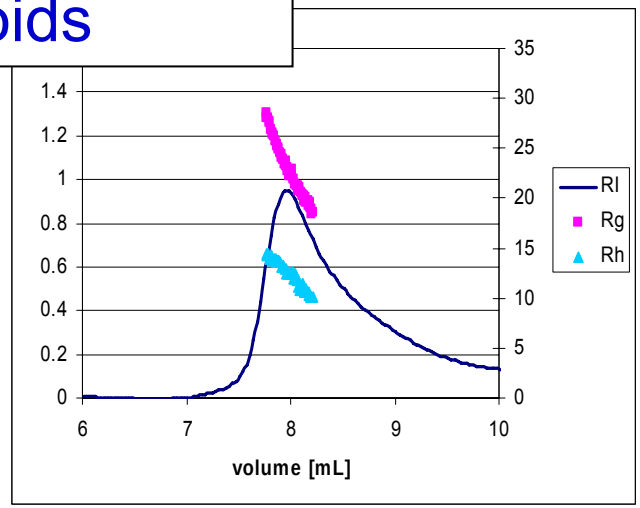
Combination of MALS (R_g) and DLS (R_h)

Ovalbumin



R_g/R_h = 0.91 Coil

Amyloids



R_g/R_h = 1.84 Rod

Shape analysis: **shape factor $\rho = R_g/R_h$**

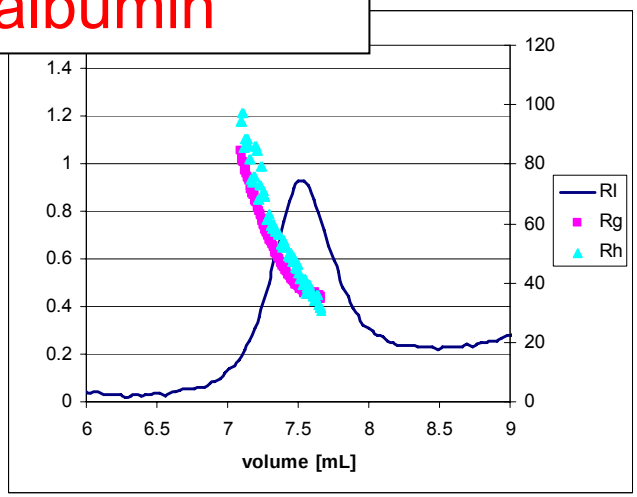
Aggregates of Ovalbumin *vs.* amyloid fibers

Shape factor: $\rho = R_g/R_h$

Combination of MALLS (R_g) and DLS (R_h)

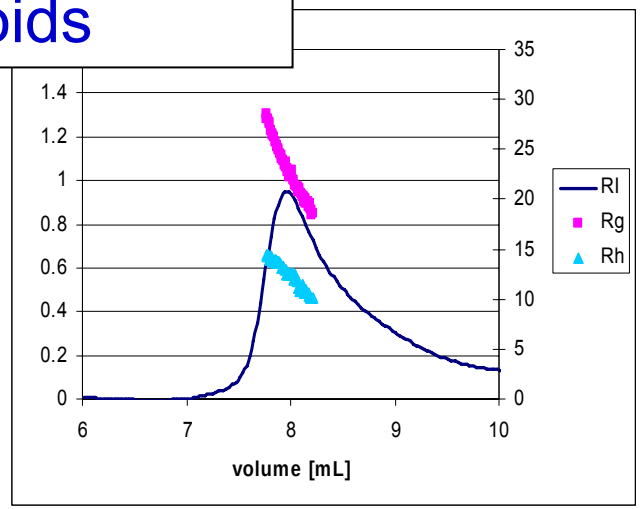
For	$\rho = R_g/R_h$
Sphere	0.774
Coil	0.816
Rod	1.732

Ovalbumin



$R_g/R_h = 0.91$ Coil

Amyloids



$R_g/R_h = 1.84$ Rod

Ova_aggr $\nu = 0.4$ Sphere/Coil

Amyloids $\nu = 0.8$ Coil/Rod

Shape analysis:

shape factor $\rho = R_g/R_h$

$\rho = R_g/R_h = 1.84$ Rod

$\log(R_g)$ *versus* log(MM) Slope = ν

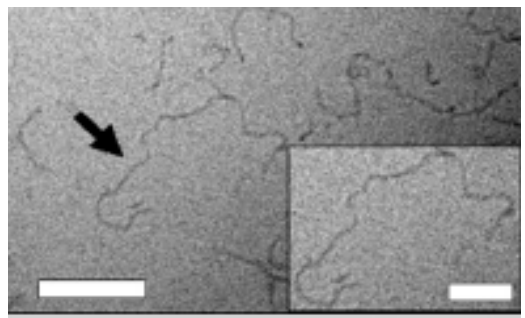
Amyloids $\nu = 0.8$ Coil/Rod

TABLE 1 Summary of scaling exponents and average ρ-ratio values [a]

	γ_c	η_c	Avg ρ–ratio	γ_m (ν)	η_m
aCgn (α-chymotrypsinogen A)	–0.3 ± 0.1	–0.27 ± 0.07	1.65 ± 0.1	0.74 ± 0.16	0.64 ± 0.12
bG-CSF (bovine granulocyte-colony stimulating factor)	–1.13 ± 0.34	–1.25 ± 0.34	1.76 ± 0.13	0.74 ± 0.15	0.8 ± 0.4

[a] Weiss W F, IV, Hodgdon T. K., Kaler E. W., Lenhoff A. M., and Roberts C. J. (2007) Nonnative Protein Polymers: Structure, Morphology, and Relation to Nucleation and Growth. *Biophysical Journal* **93**: 4392-4403

Cryo-TEM micrograph of aCgn samples (c_0 = 1 mg/mL) at m = 0.05



Weiss W F, IV, Hodgdon T. K., Kaler E. W., Lenhoff A. M., and Roberts C. J. (2007) Nonnative Protein Polymers: Structure, Morphology, and Relation to Nucleation and Growth. *Biophysical Journal* **93**: 4392-4403

Determination of dimerization constant from SEC-LS measurements

SecA protein

WT	monomer =	102 kDa
DS8 deletion mutant	monomer =	101 kDa
D11 deletion mutant	monomer =	100 kDa

SecA protein

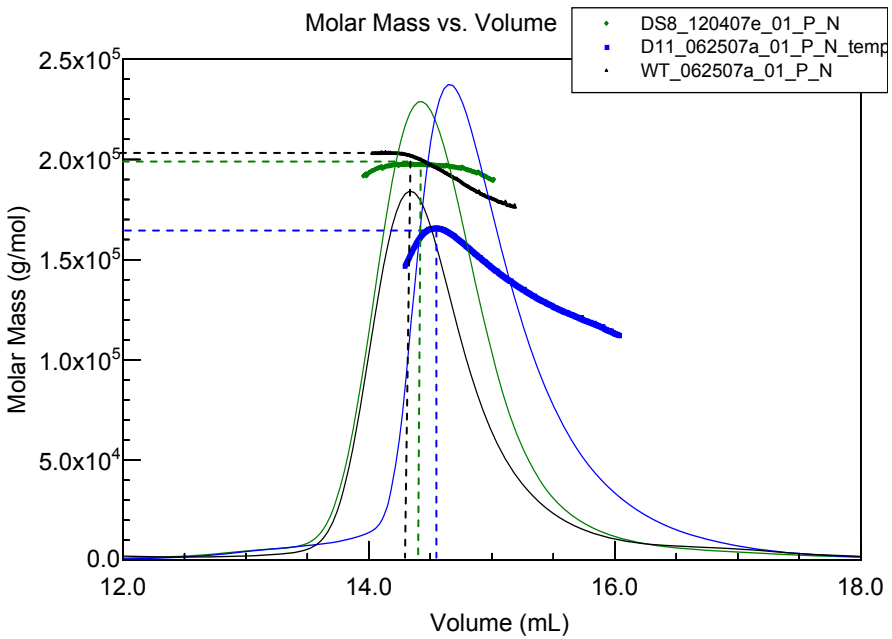
WT monomer = 102 kDa

DS8 deletion mutant monomer = 101 kDa

D11 deletion mutant monomer = 100 kDa

Low salt buffer:

10 mM Tris pH 7.5, 5 mM Mg2+, 100 mM KCl



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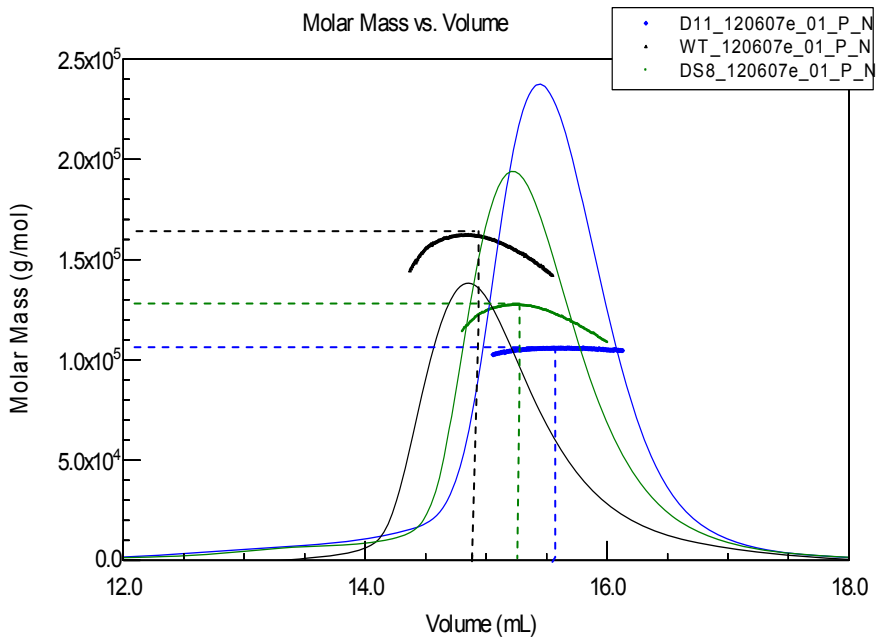
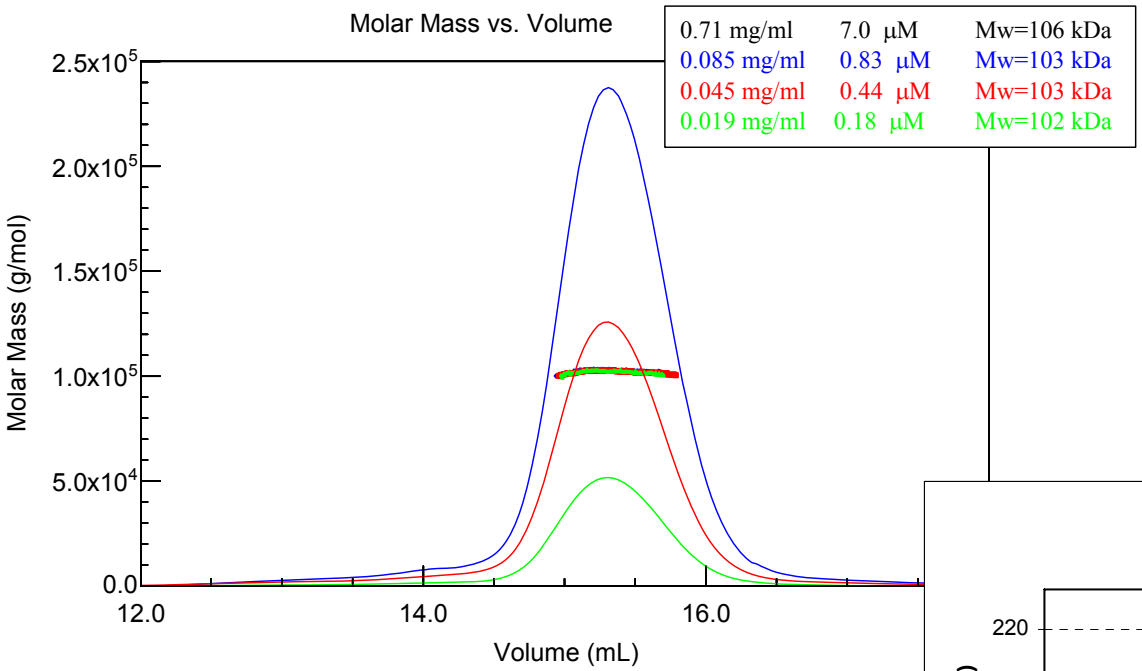
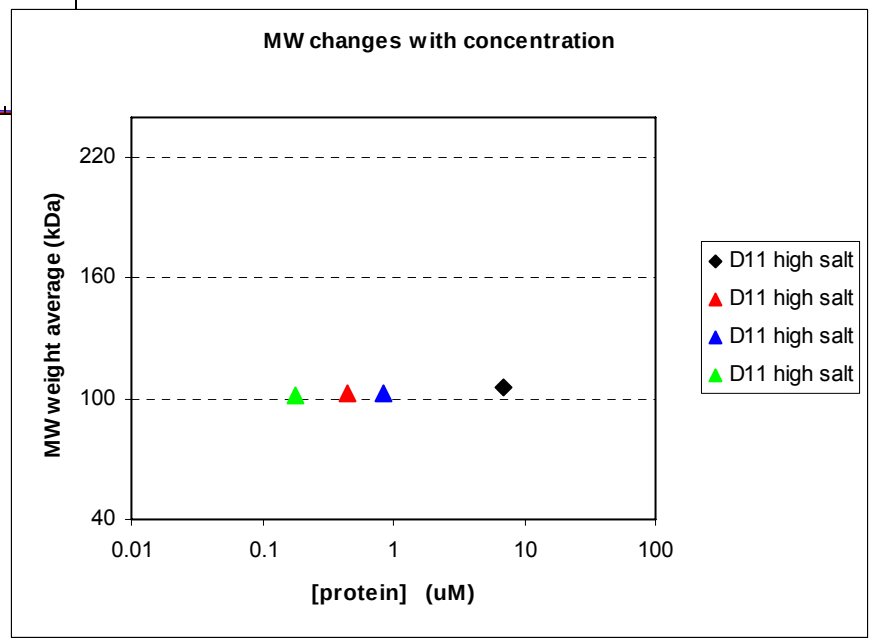


D11 deletion mutant
mono= **101 kDa**

High salt buffer:

10 mM Tris pH 7.5, 5 mM Mg2+, 300 mM KCl,

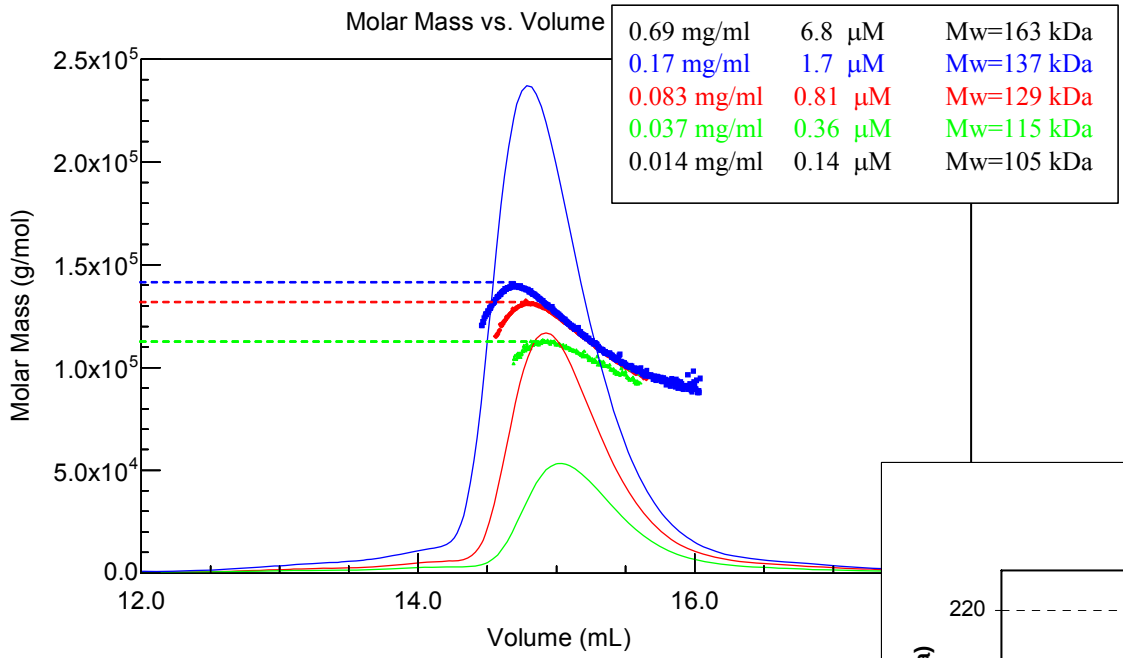


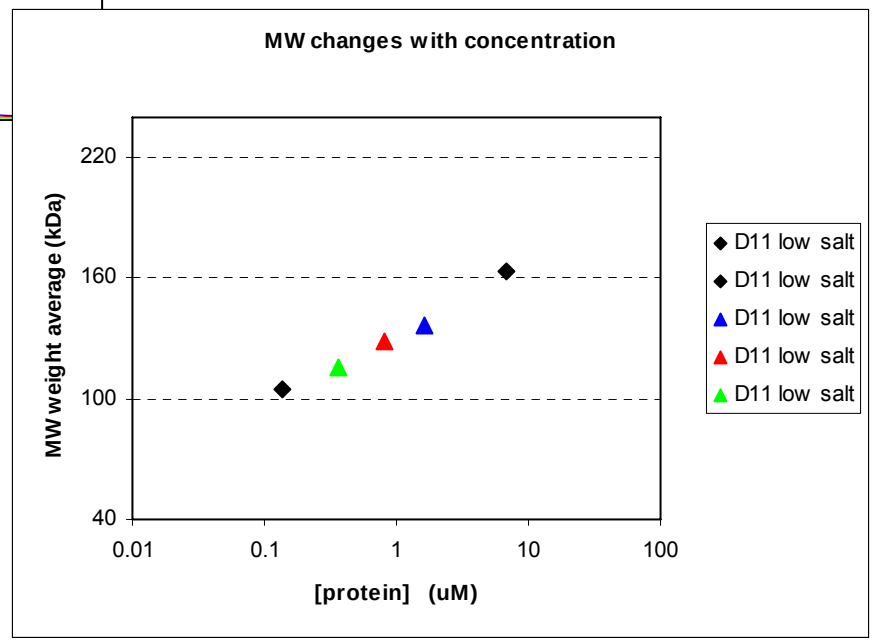


D11 deletion mutant
mono= **101 kDa**

Low salt buffer:

10 mM Tris pH 7.5, 5 mM Mg2+, 100 mM KCl,





$$M_w = f_m M_m + f_d M_d = M_m(2 - f_m)$$

$$2M = D$$

$$K_a = \frac{[D]}{[M]^2} = \frac{(1 - f_m)}{2(f_m)^2 c_t}$$

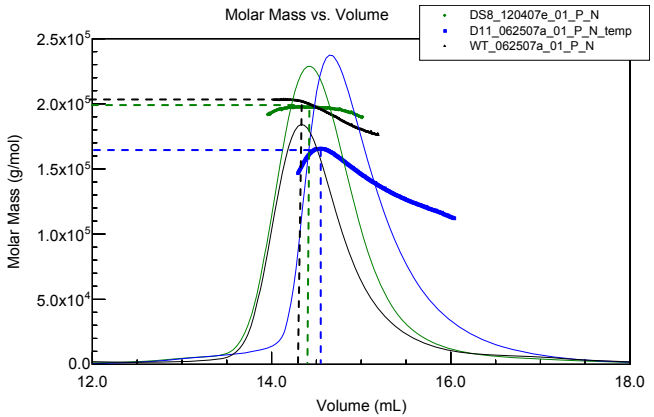
$$f_m = \frac{-1 + \sqrt{1 + 8K_a c_t}}{4K_a c_t}$$

WT monomer = 102 kDa

DS8 deletion mutant monomer = 101 kDa

D11 deletion mutant monomer = 100 kDa

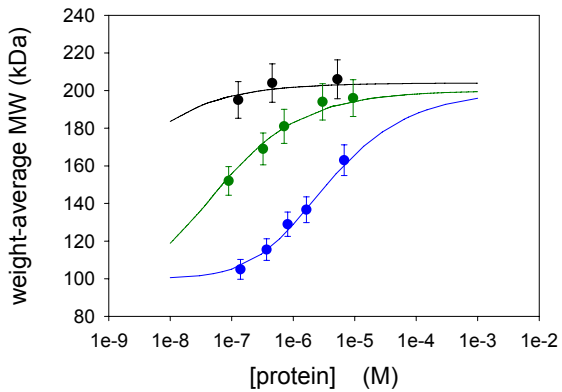
Low salt buffer: 100 mM KCl



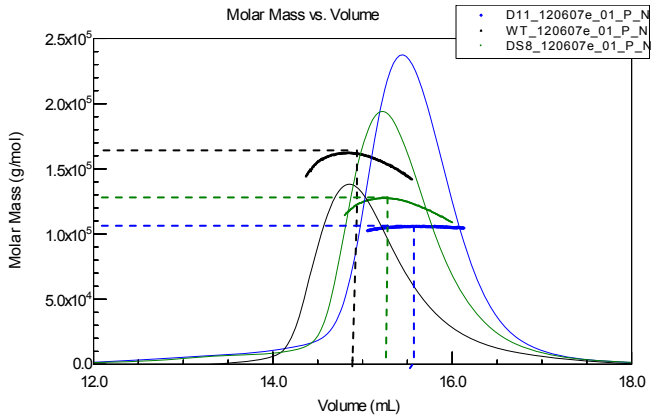
WT Kd= <1e-9

DS8 Kd=7±1e-8 M

D11 Kd=3.5±0.2e-6 M



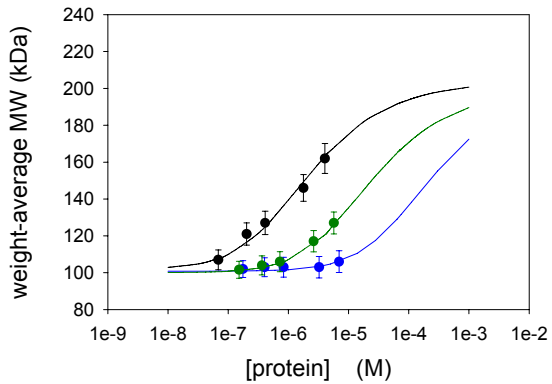
High salt buffer: 300 mM KCl



WT Kd= 2.2±0.2e-6 M

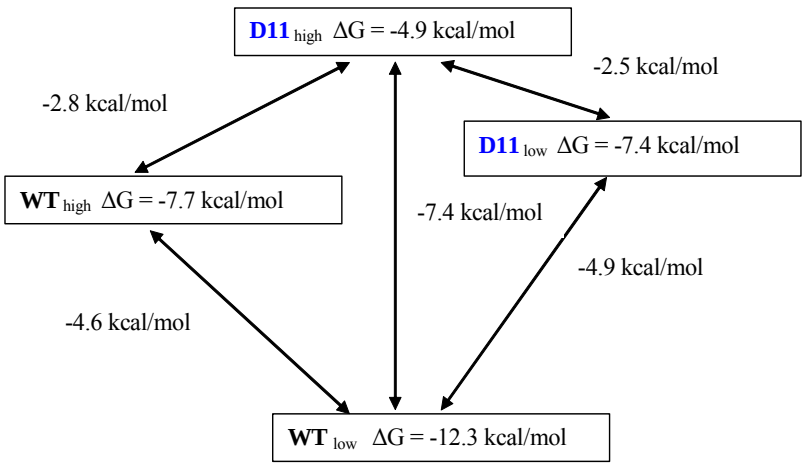
DS8 Kd= 2.41±0.05e-5 M

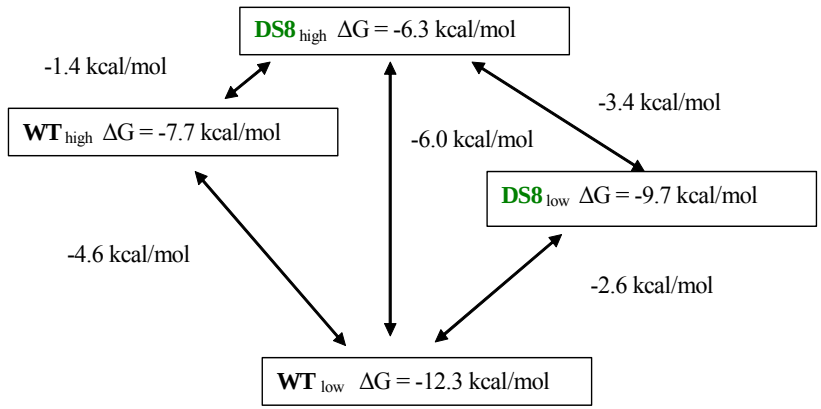
D11 Kd> 2.4e-4 M



Thermodynamic linkage for SecA dimerization

Protein	Low Salt 100 mM KCl		High Salt 300 mM KCl	
	Kd [M]	ΔG dimer (kcal/mol)	Kd [M]	ΔG dimer (kcal/mol)
WT	$<1\times10^{-9}$	-12.3	$2.2\pm0.2\times10^{-6}$	-7.7
DS8	$7\pm1\times10^{-8}$	-9.7	$2.41\pm0.05\times10^{-5}$	-6.3
D11	$3.5\pm0.2\times10^{-6}$	-7.4	$>2.4\times10^{-4}$	-4.9





Static LS

- fast and accurate determination of molar masses (weight average)
  - glycosylated protein, conjugated with PEG, protein-lipids-detergent complexes, protein-nucleic acid complexes
- accuracy of ± 5% in molar mass determination
- easy to implement, fully automated (data collection and data analysis)
- highly reproducible (no operator's bias)
- SEC/MALS excellent in detecting and quantifying population with various oligomeric state in protein
- can be used to determine association constant (concentration gradient measurements)

Combined data about MM, Rg and Rh - shape information (multiangle static and dynamic LS)

- via frictional ratio (shape factor) R_h/R_s
- via shape factor ν, from $\log(R_g)$ vs. $\log(MM)$ plot
- via shape factor ρ, from R_g/R_h ratio

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NIH

Users of SEC/LS Service

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