BioProcess International™

Analytical and Quality Summit

Application of Light Scattering Techniques for Analysis of Oligomerization and Particle Formation

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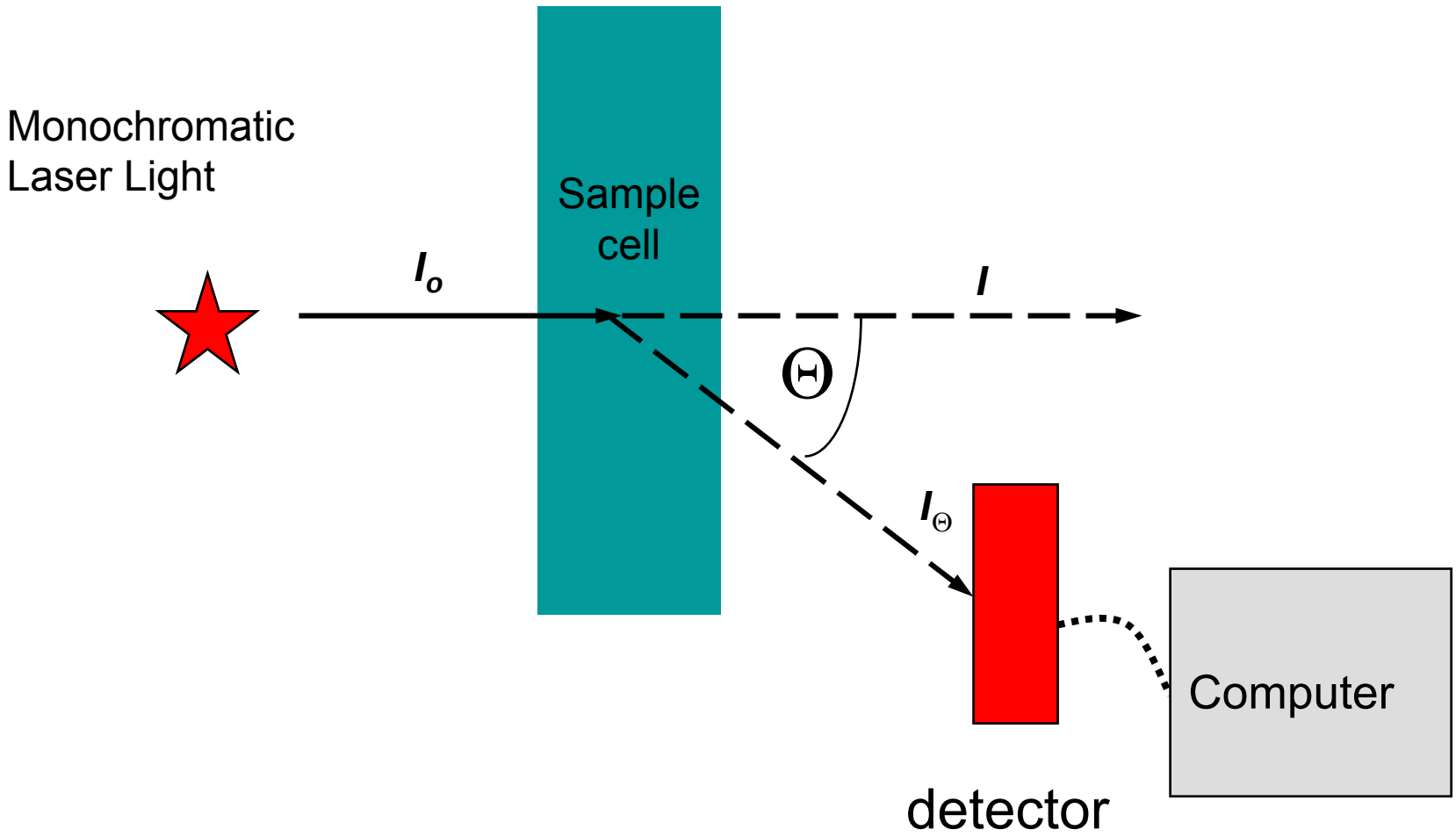
Outline

- Light Scattering Technologies
  - Static and dynamic light scattering
  - Parameters derived from SLS and DLS measurements
- Batch Light Scattering Applications
  - Detection of aggregates in DLS and SLS measurement
- Flow Mode Light Scattering Applications
  - Molar mass distributions and differences in populations
  - Characterization of morphology of aggregates
- Determination of an oligomeric state of modified proteins from SEC-LS/UV/RI measurement
- Capabilities and limitation of static and dynamic LS measurements

Light Scattering Experiments



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 <u>hydrodynamic</u> radius (Stokes radius)

Uncertainty of ~10% for monodisperse sample

Rayleigh-Debye-Zimm formalism

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

R(Θ)	Rayyleigh ratio (excess scattered light)
c	sample concentration (g/ml)
Mw	weight-average molecular weight (molar mass)
A2	second virial coefficient (ml-mol/g2)
P(Θ)	form factor (angular dependence)

Stokes-Einstein

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

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

Why Light Scattering?

- Scattering Intensity, $R(\Theta) \sim Mw*c$

  because of their big Mw, aggregates scatter strongly even when present at low concentrations; easily detectable

- Angular variation of the scattered light is related to the size of the molecule

  the light scattering signal from aggregates will show angular dependence, while LS signal produces by lower order oligomers like dimers, trimers, tetramers, et c. will not

- LS measurements are non-invasive and non-destructive

  - small sample volumes
  - great dynamic range for sizing: hydrodynamic radii ~ 2nm to 500 nm
  - great dynamic range for Mw determination: < 1kDa to >10 MDa
  - wide range of concentrations (non-ideality can be addressed through the determination of second virial coefficient)
  - perfectly suited for determination of oligomeric state of modified proteins without prior knowledge of extend of modificaqtion (glycosylated, modified by polyethylene glycol, or membrane proteins present as complexes with lipids and detergents

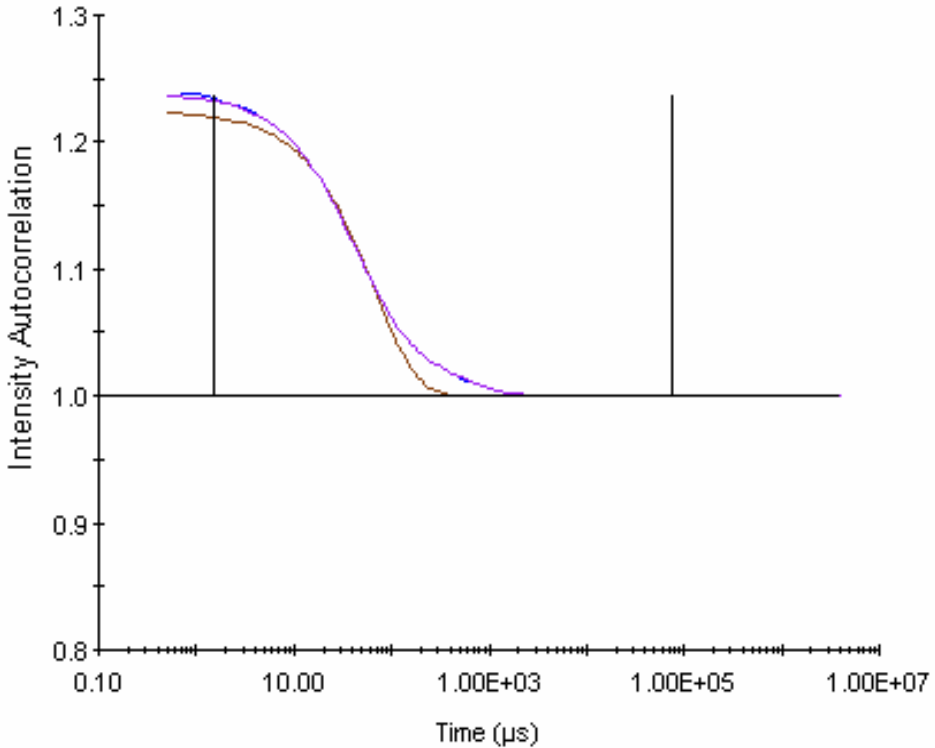
Determination of hydrodynamic radius, R_h, from a Dynamic LS experiment

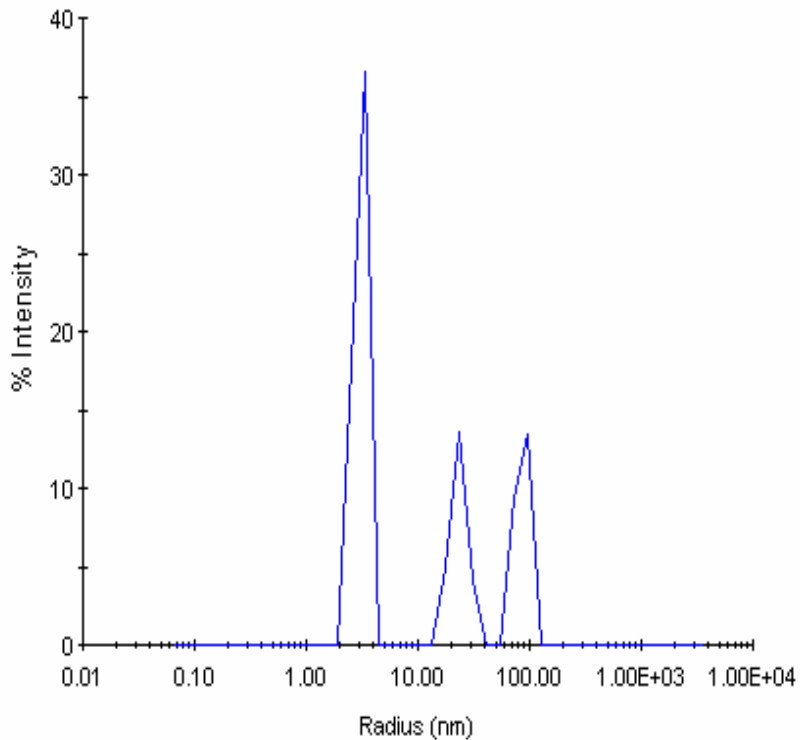
Ovalbumin; monomer: 43 kDa; Rh=3.0 nm

Rh = 8±7 nm from Cumulant Fit (Polydispersity 93%)

Regularization Fit:

Peak	Rh (nm)	Polydispersity (%)	MW (R) kDa	% Intensity	% Mass
1	3.1	12.8	46	54	99.9
2	24	17.8	>1MDa	23	0.1
3	86	13.4	>1MDa	23	<0.1





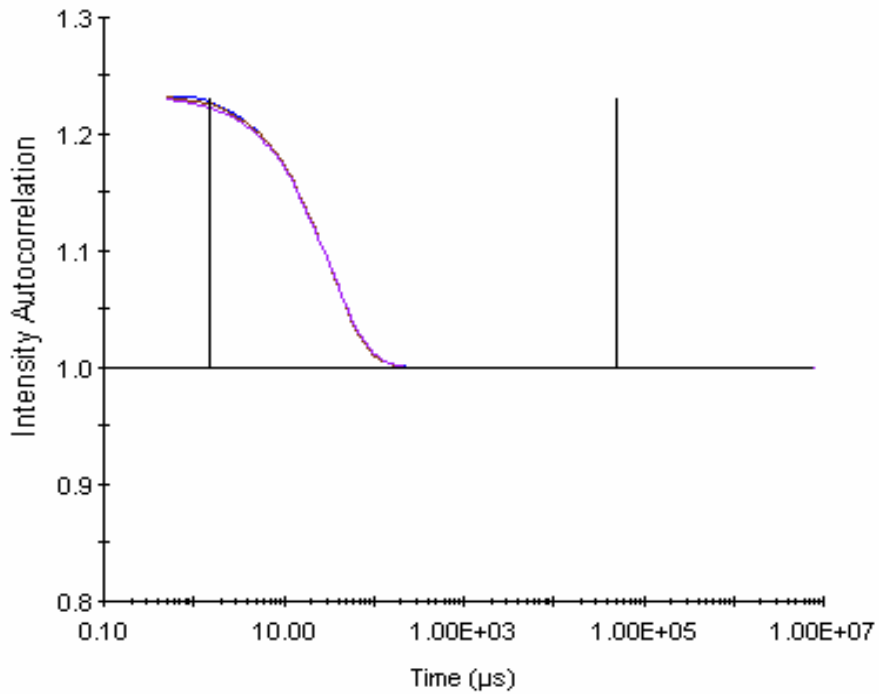
Results from a batch mode Dynamic LS experiment:

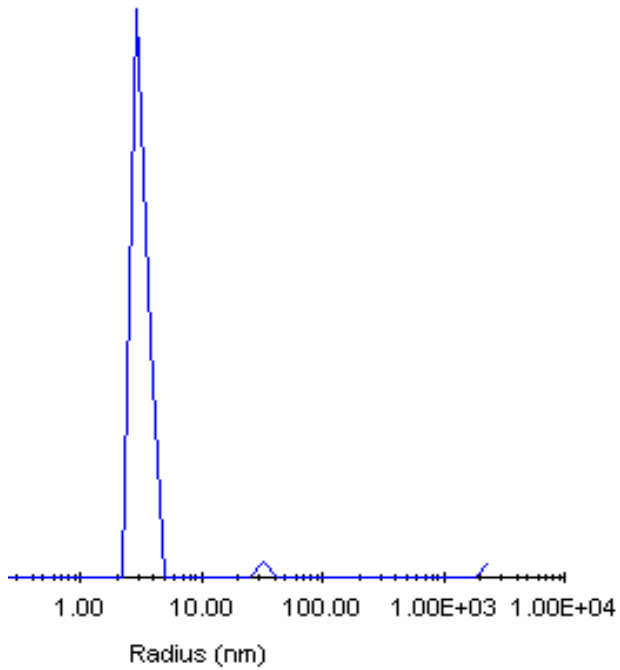
Ovalbumin 43 kDa; Rh=3.0 nm

Rh = 3.2±0.6 nm from Cumulant Fit (Polydispersity 19%)

Regularization Fit:

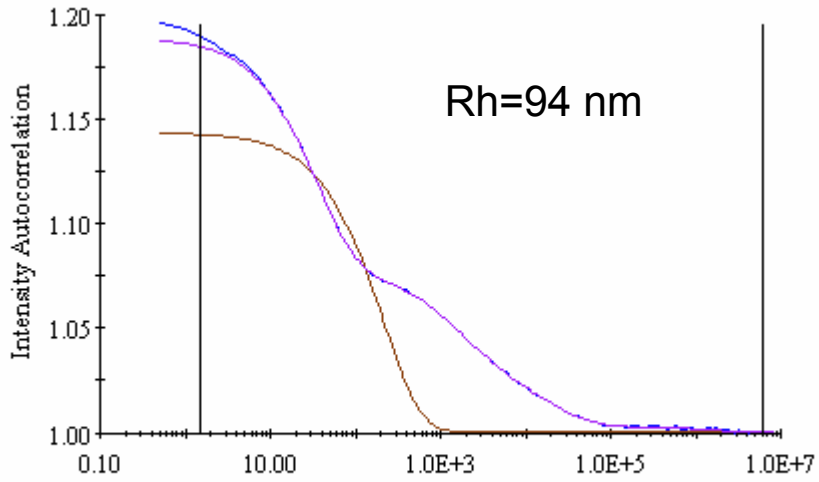
Peak	Rh (nm)	Polydispersity (%)	MW (R) kDa	% Intensity	% Mass
1	3.1	12.9	46	96	100
2	32	0	>1MDa	2	0
3	2423	0	>1MDa	2	0

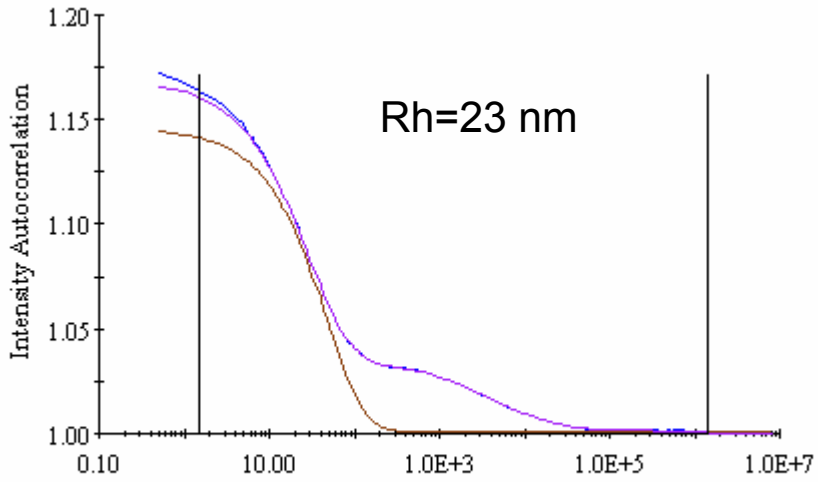


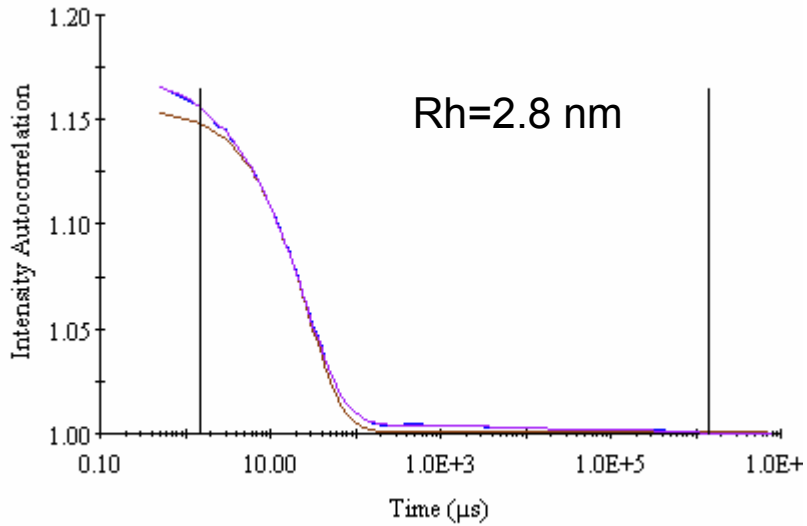


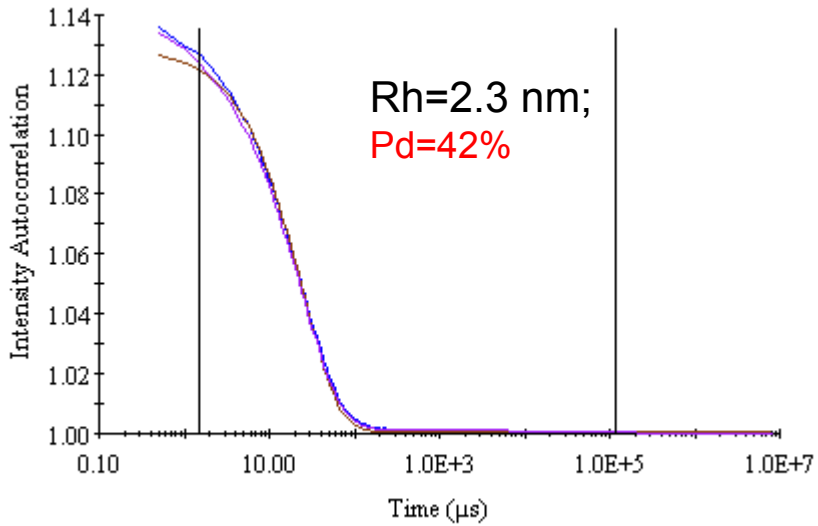
Dissociation of aggregates upon dilution; time course

Protein H 23 kDa; Rh=2.3 nm



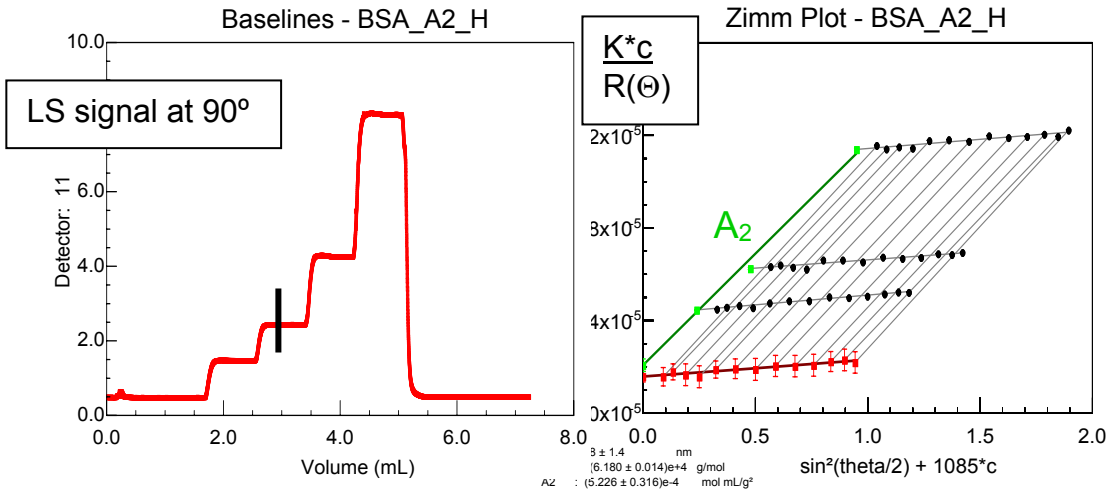






Determination of Molar Mass and second virial coefficient from a batch static LS experiment

BSA 66 kDa



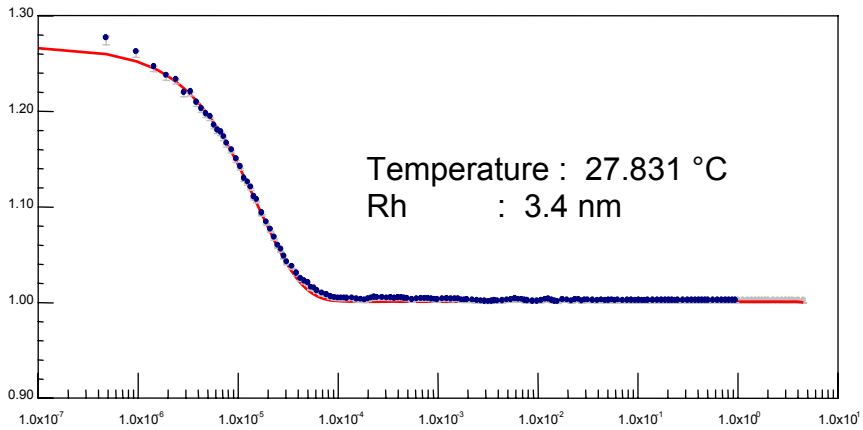
$$\frac{K^*c}{R(\theta)} = \frac{1}{MwP(\theta)} + 2A_2c$$

and Rh from DLS

Zimm plot analysis of static light scattering data

Mw = 62 kDa

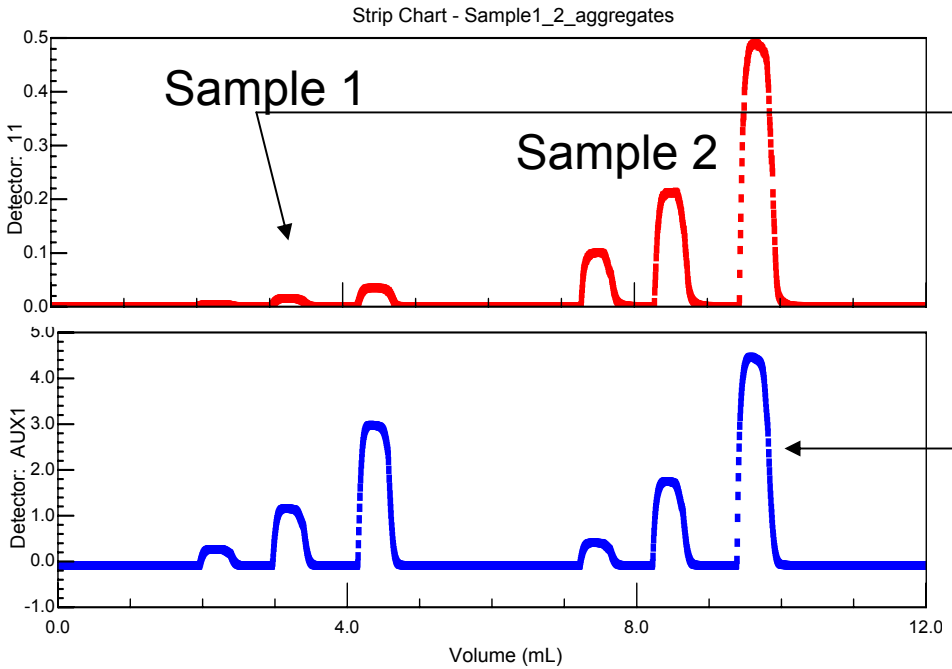
A_2 = (5.226 ± 0.316)e^{-4} mol mL/g²

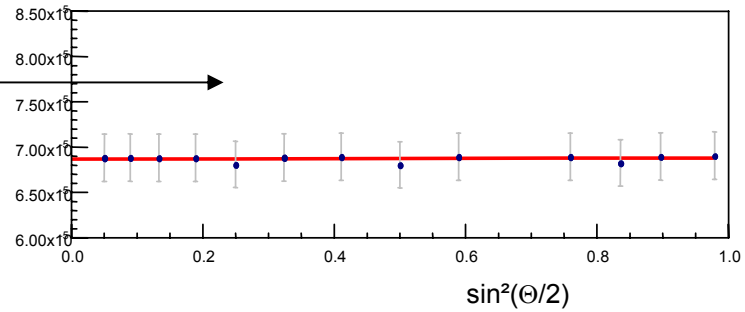


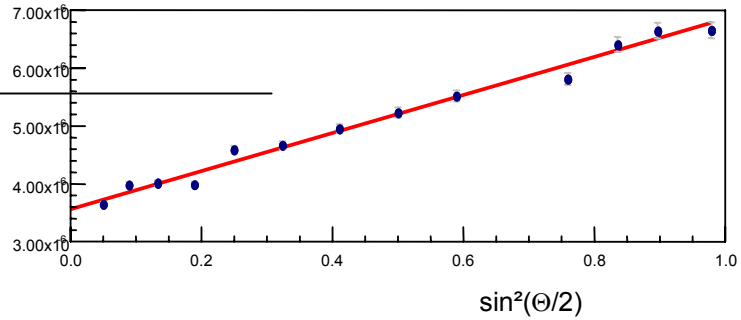
B. H. Zimm, (1948) J. Chem. Phys., 16, 1099

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

Feature detected in a batch mode LS measurements for sample containing aggregates

- Static (classical)

Aggregates present:

- elevated weight average Molar Mass (M_w weight average)
- angular dependence in scattered light

- Dynamic (quasielastic)

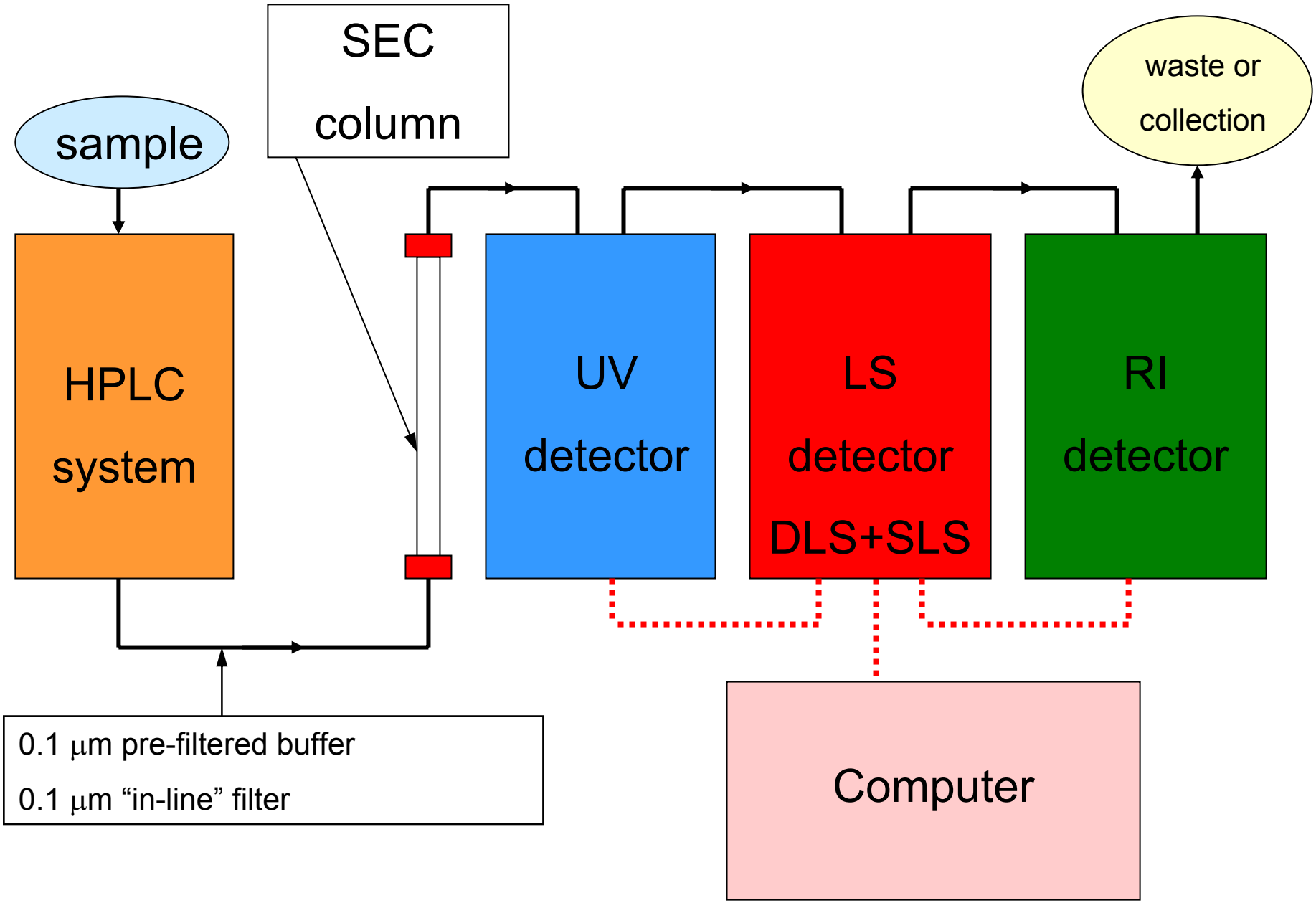
Aggregates present:

- autocorrelation function cannot be described by single exponential (cumulant fit)
- polydispersity from cumulant fit >15%

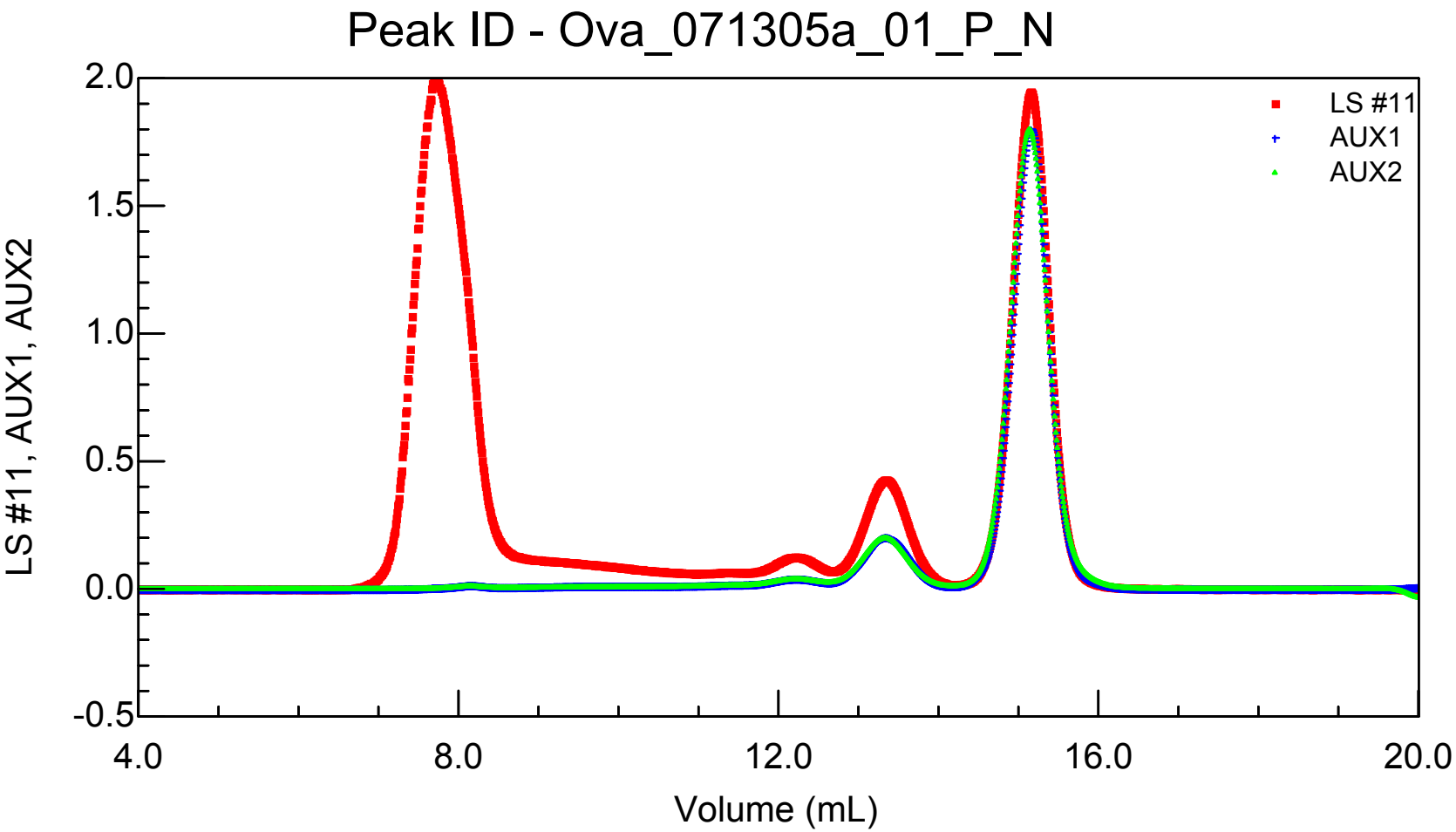
Missing information: how much and what size?

Solutions

- Sample fractionation followed by batch measurements
- **Column separation with simultaneous LS characterization**


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

Three Detector monitoring



UV at 280 nm

RI

LS at 90°

Ovalbumin 43 kDa

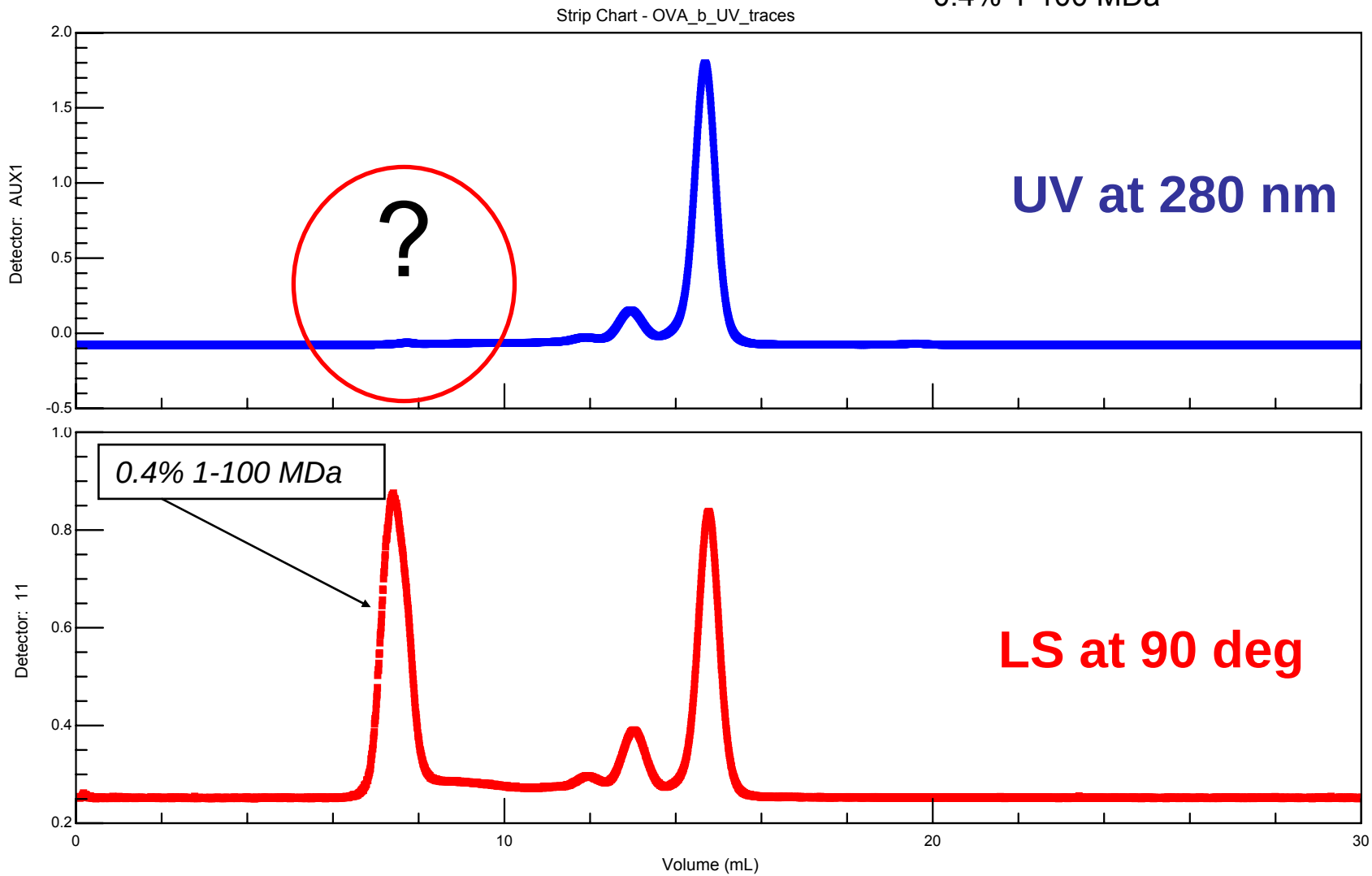
88% monomer

8% dimer

1.5% trimer

3% aggregates < 1MDa

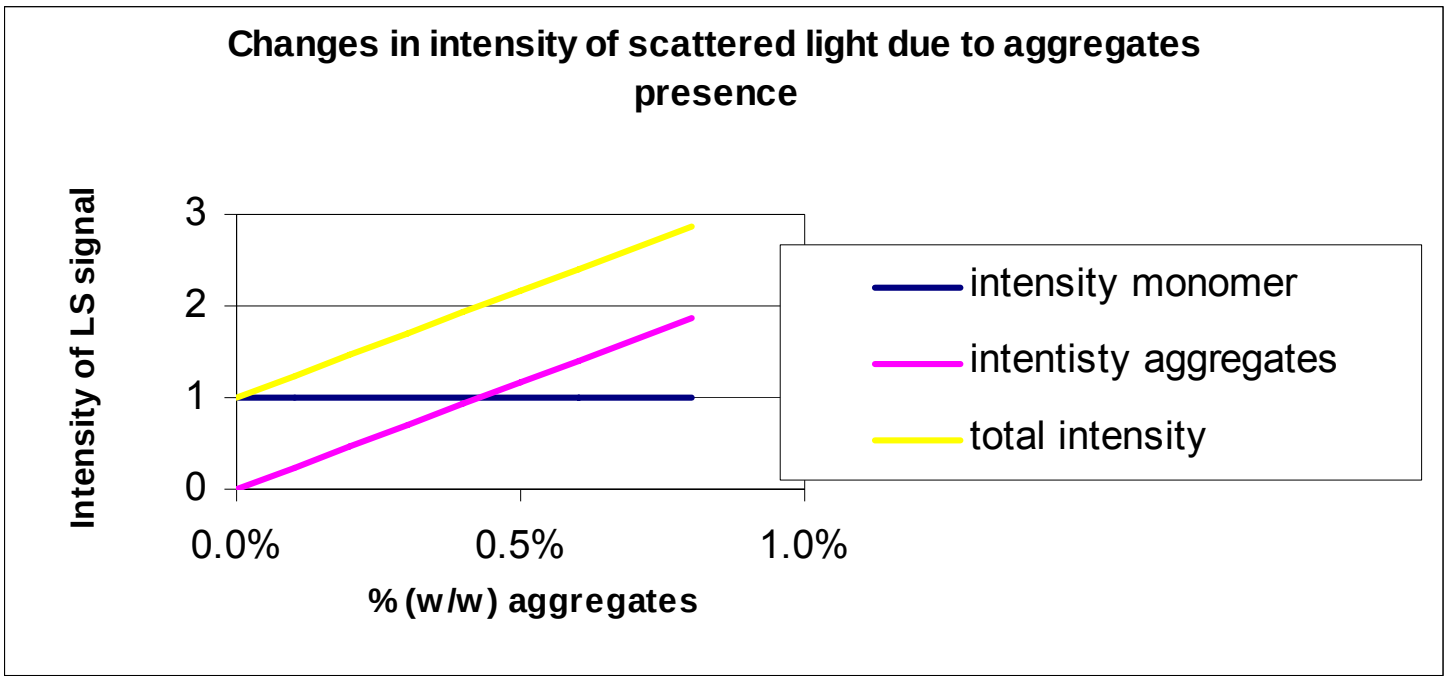
0.4% 1-100 MDa



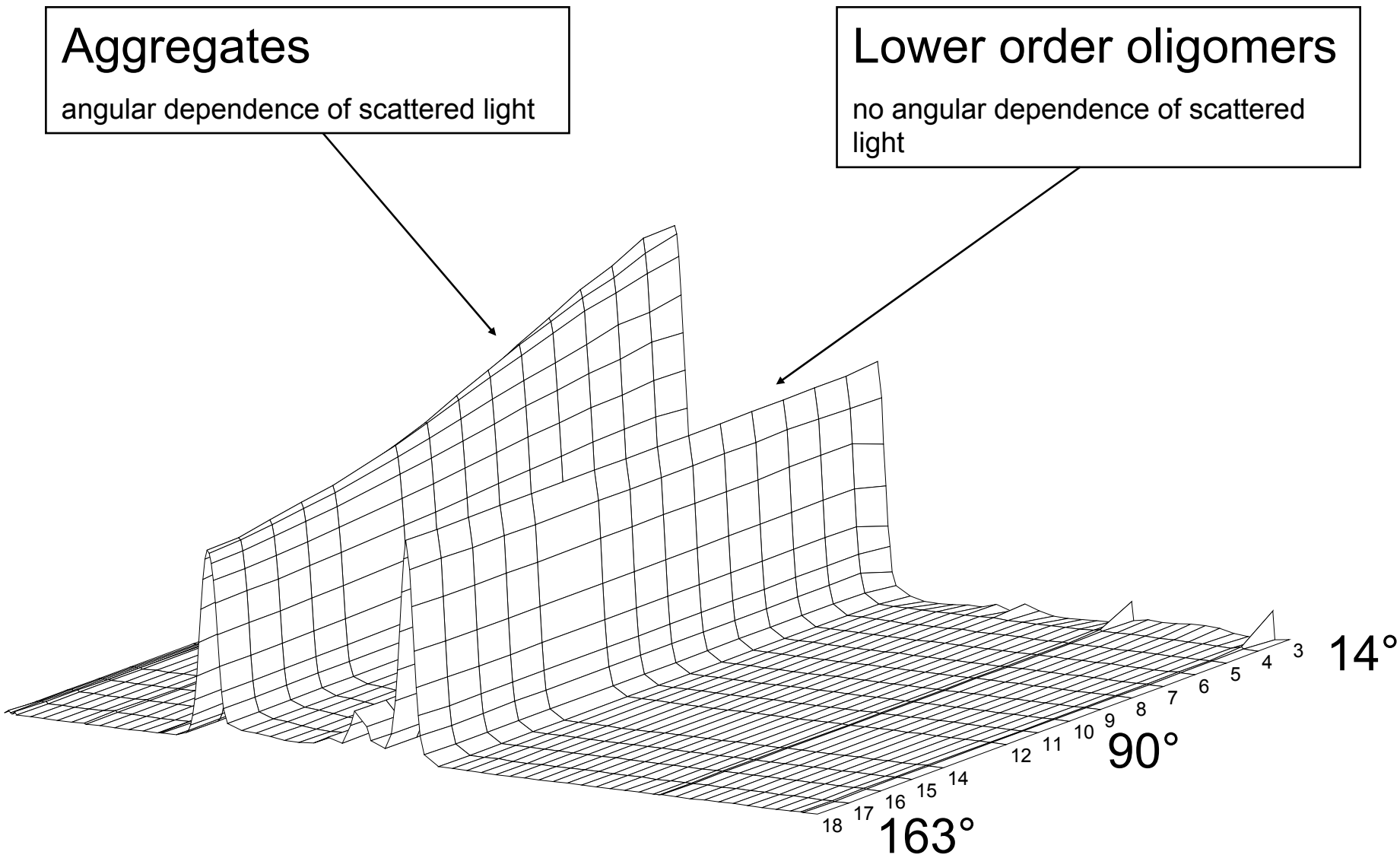
Intensity of scattered light ~ M_w*c

due to their high Mw aggregates scatter very strongly

A monomeric protein 43 kDa and aggregates 10 MDa at 2 mg/mL:

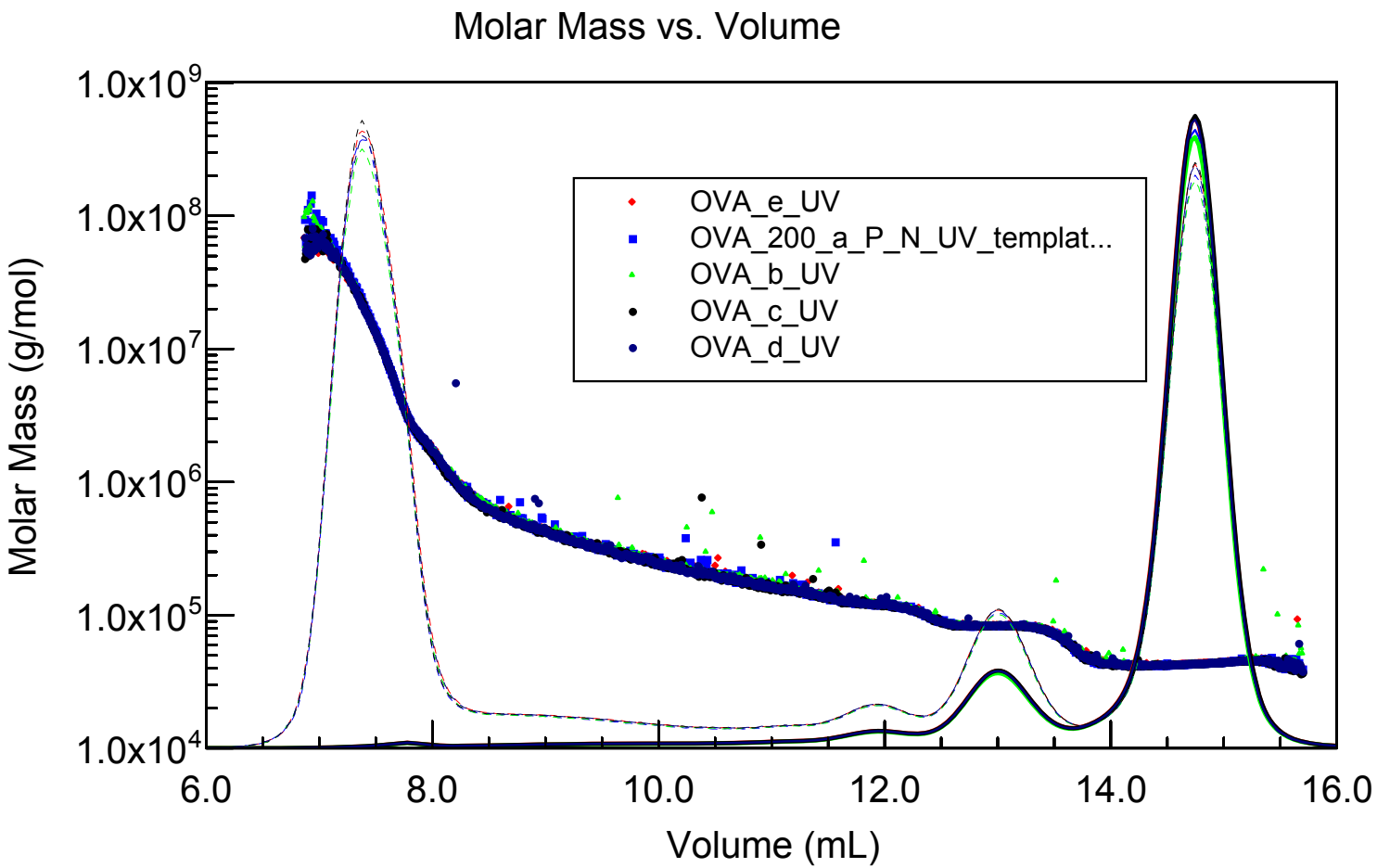


Ovalbumin 43 kDa

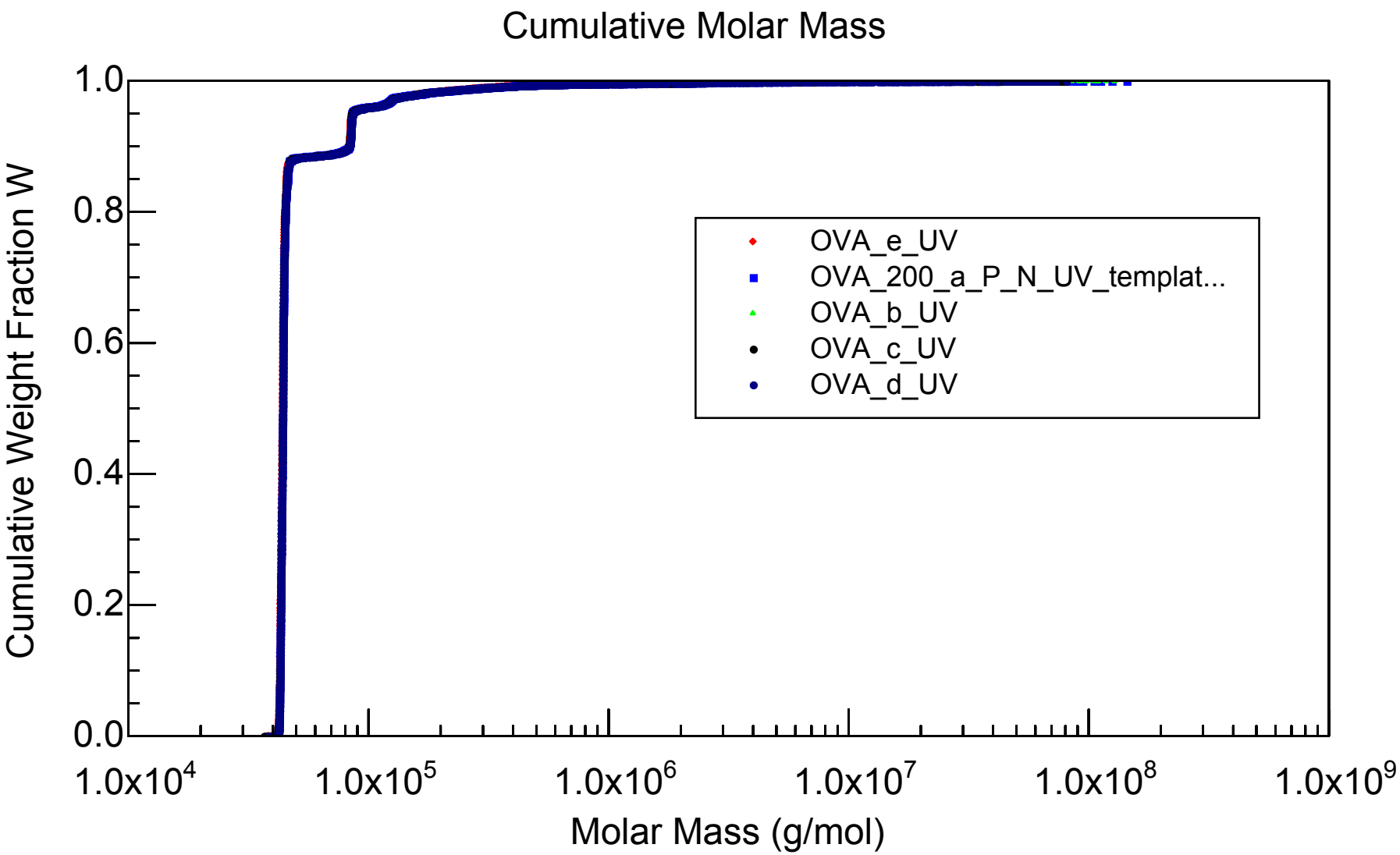


Molar mass distribution for multiple analyses

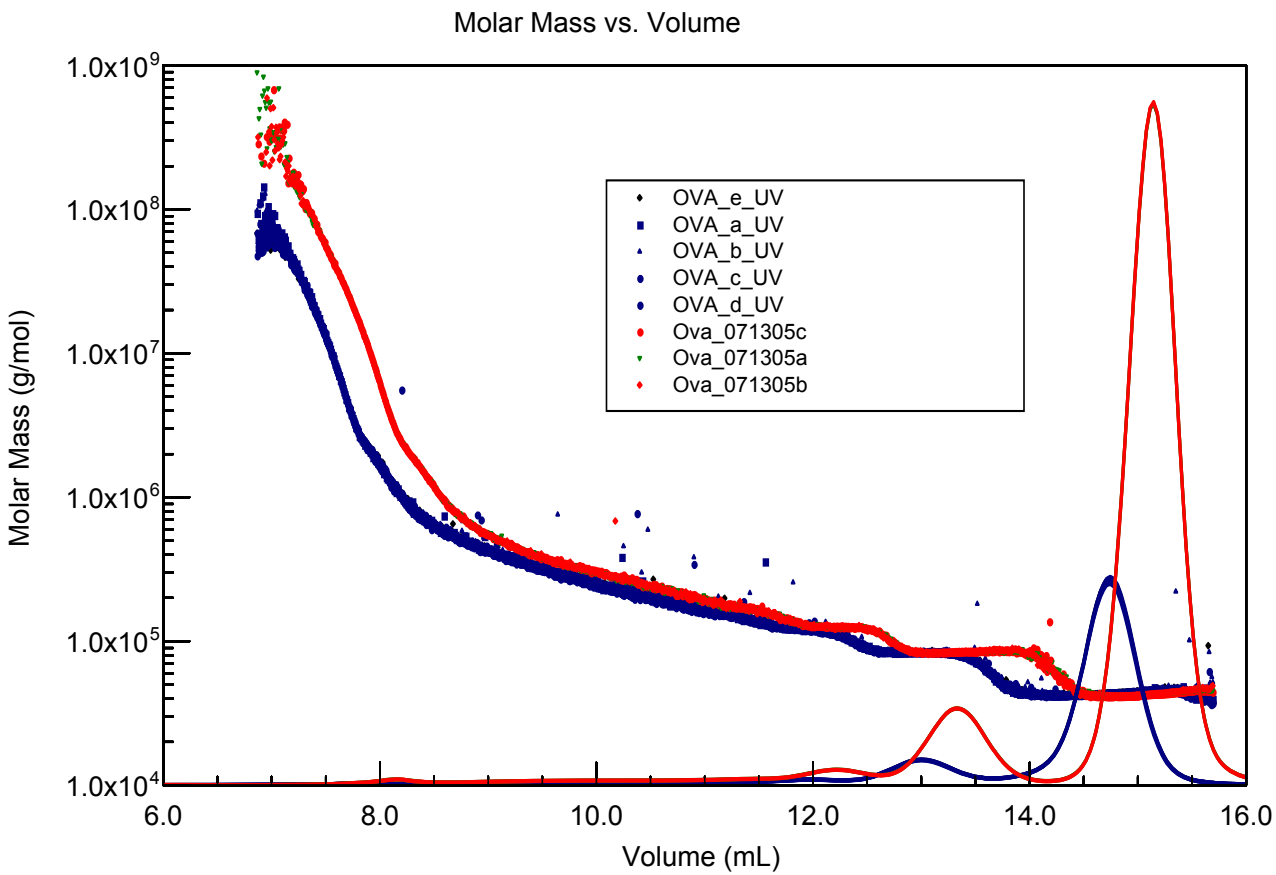
Ovalbumin 43 kDa automated template processing of five data sets



Determination of Weight Fractions



Differences in population based on molar mass distribution



Ovalbumin (5 runs)

Mw = 108 ± 17 kDa

Polydispersity Mw/Mn

2.3 ± 0.4

Ovalbumin (3 runs)

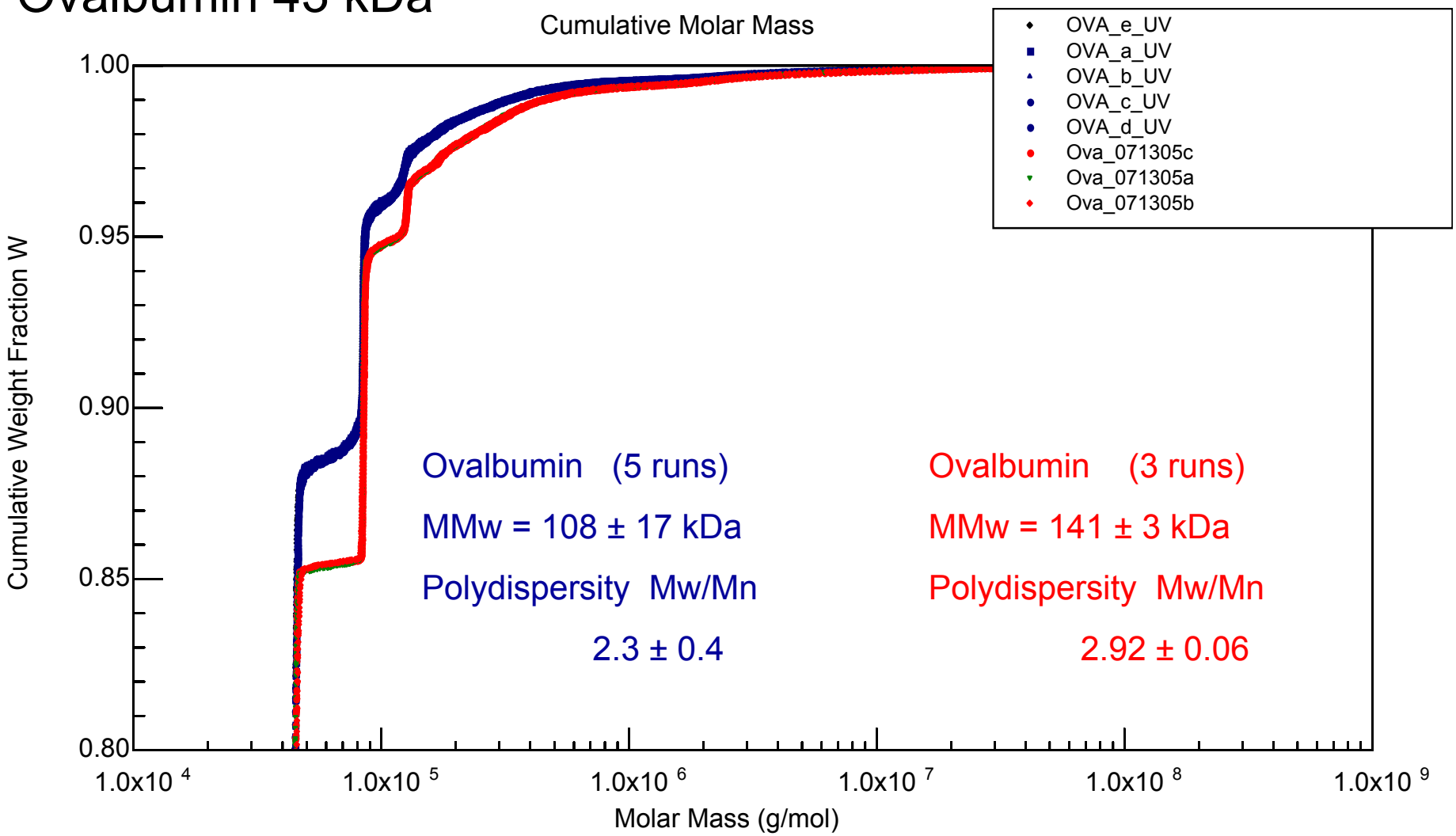
Mw = 141 ± 3 kDa

Polydispersity Mw/Mn

2.92 ± 0.06

Differences in population based on molar mass distribution

Ovalbumin 43 kDa



Differences in population based on molar mass distribution

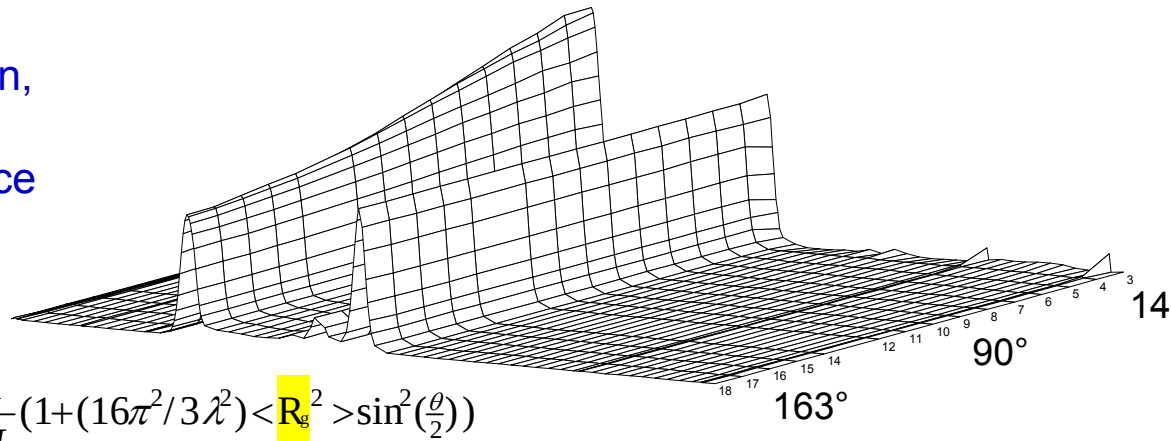
Ovalbumin 43 kDa

Oligomeric state	Average Mw ± SD [kDa] (5 analyses)	Average Mw ± SD [kDa] (3 analyses)	Fraction of Mass [% of total] (5 analyses)	Fraction of Mass [% of total] (3 analyses)
	Mw = 108 ± 17	**Mw = 141 ± 3**	**Mw = 108 ± 17**	**Mw = 141 ± 3**
Mono (20-50 kDa)	43.0 ± 0.1	42.80 ± 0.02	88.1 ± 0.1	85.23 ± 0.06
Di (50-96 kDa)	82.7 ± 0.4	84.1± 0.2	7.68 ± 0.04	9.4 ± 0.0
Tri (96-130 kDa)	114 ± 4	121.8 ± 0.7	1.54 ± 0.05	1.9 ± 0.0
Agg. (0.13 –1 MDa)	270 ±10	284 ± 2	2.18 ± 0.08	2.87± 0.06
Agg. (1 –100 MDa)	10±1 x10^3	10.9±0.4 x10^3	0.4 ± 0.0	0.6 ± 0.0

Morphology of aggregates from angular dependence of LS signal;

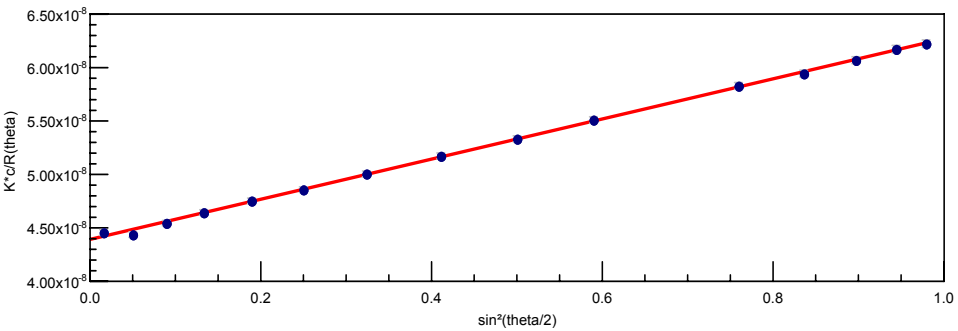
size determination- Rg

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



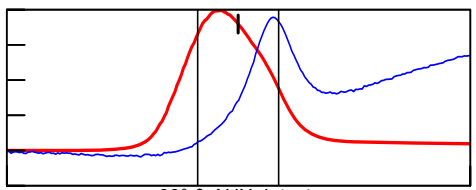
Zimm Plot

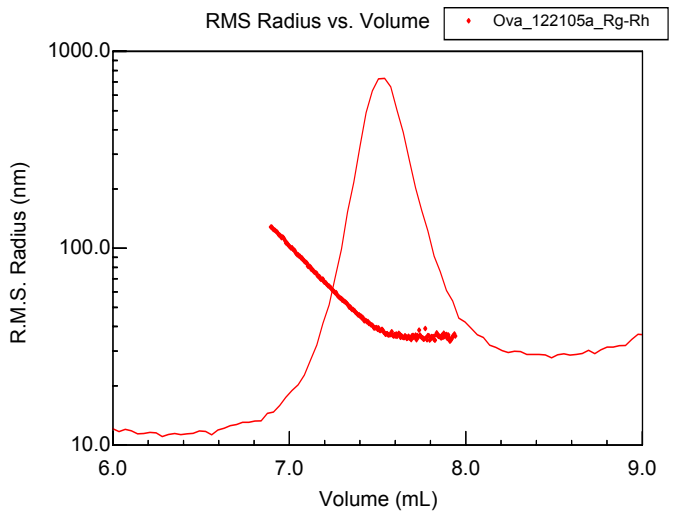
$$\frac{K^*c}{R(\theta)} = \frac{1}{M_w}\left(1+\left(16\pi^2/3\lambda^2\right)\langle R_g^2\rangle \sin^2\left(\frac{\theta}{2}\right)\right)$$



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





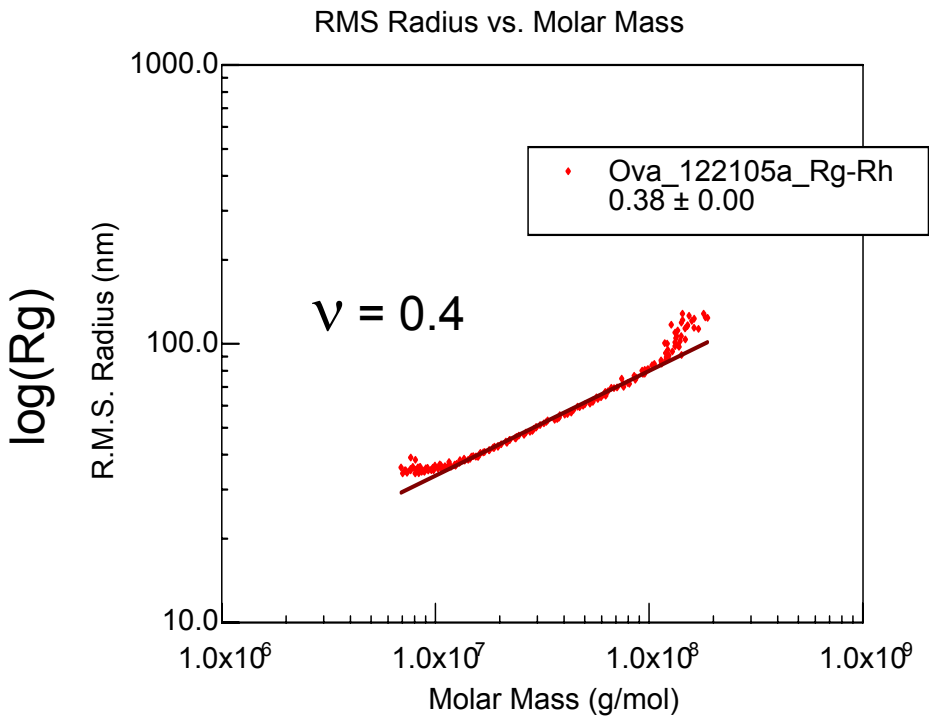
Inferring conformational information from the relationship between molecular size (Rg) and molecular weight (Molar Mass)

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

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

Slope = ν

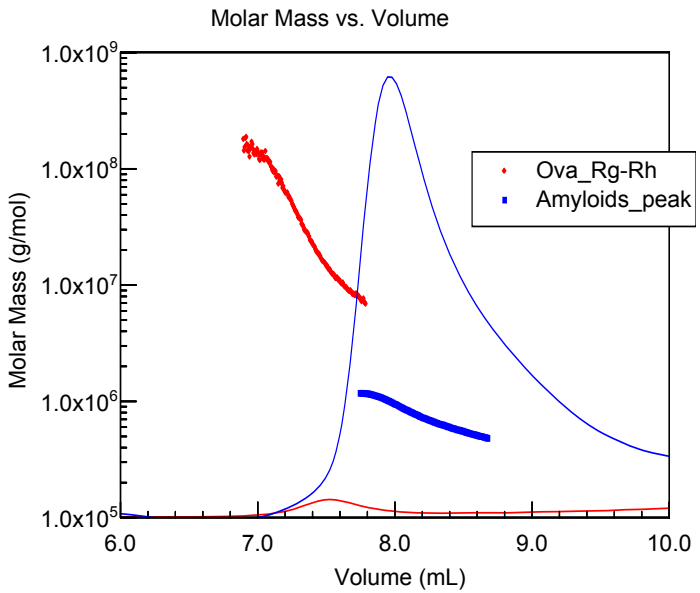
For	ν
Sphere	0.33
Coil	0.5
Rod	1

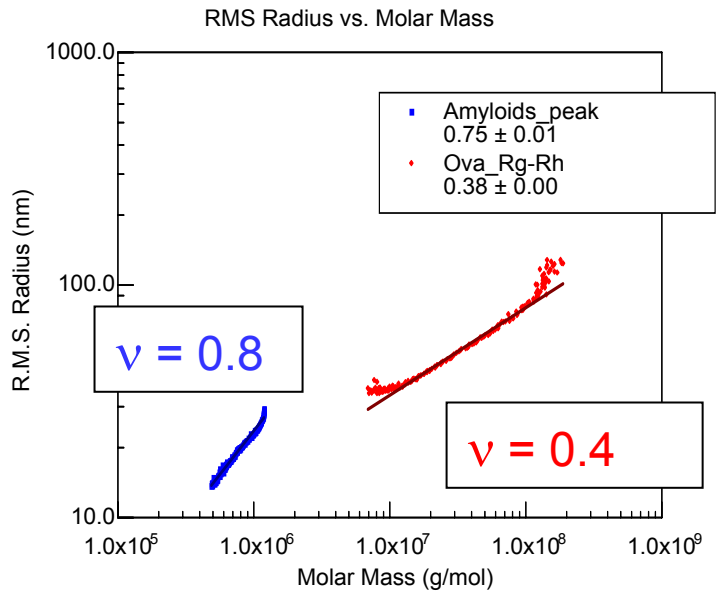


Rollings, J.E. (1992) in *"Laser Light Scattering in Biochemistry"*, Eds. S.E. Harding, D. B. Sattelle and V. A. Bloomfield; p. 275-293

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 $\rho = R_g/R_h$**

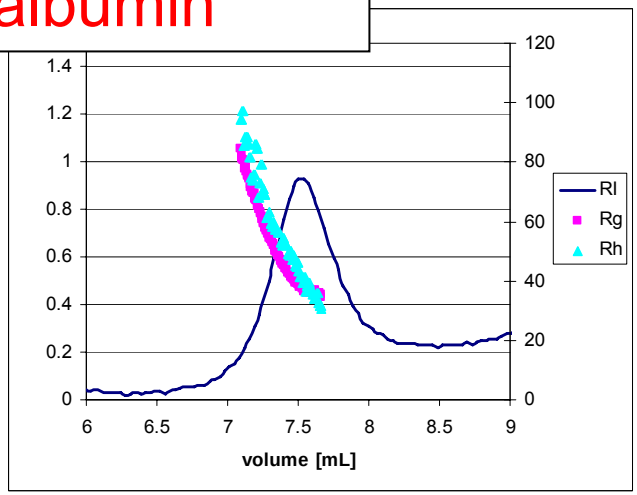
Aggregates of Ovalbumin *vs.* amyloid fibers

Shape factor: $\rho = R_g/R_h$

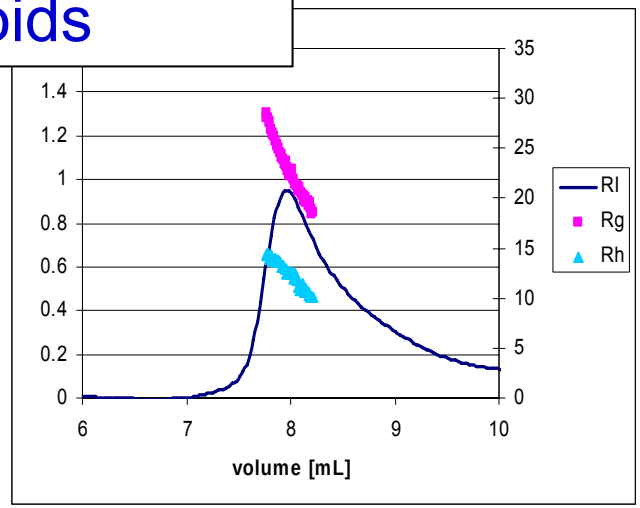
For	$\rho = R_g/R_h$
Sphere	0.774
Coil	0.816
Rod	1.732

Combination of MALS (R_g) and DLS (R_h)

Ovalbumin



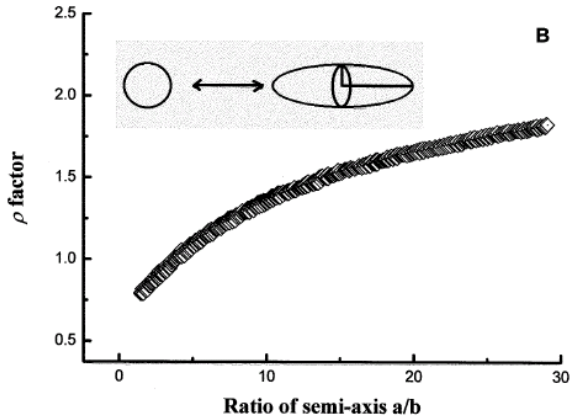
Amyloids



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

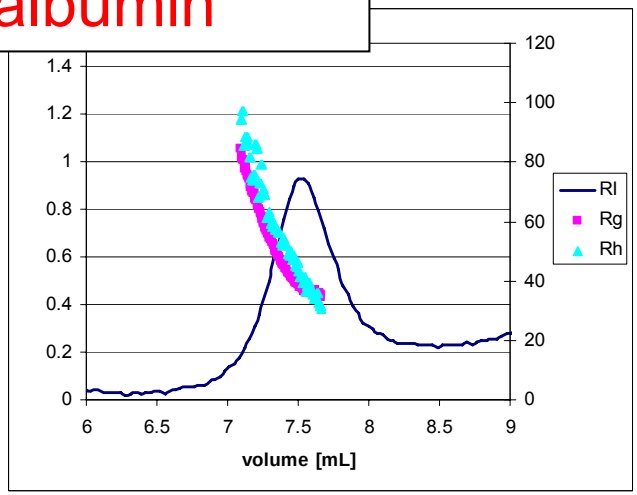
Aggregates of Ovalbumin *vs.* amyloid fibers

Shape factor: $\rho = R_g/R_h$

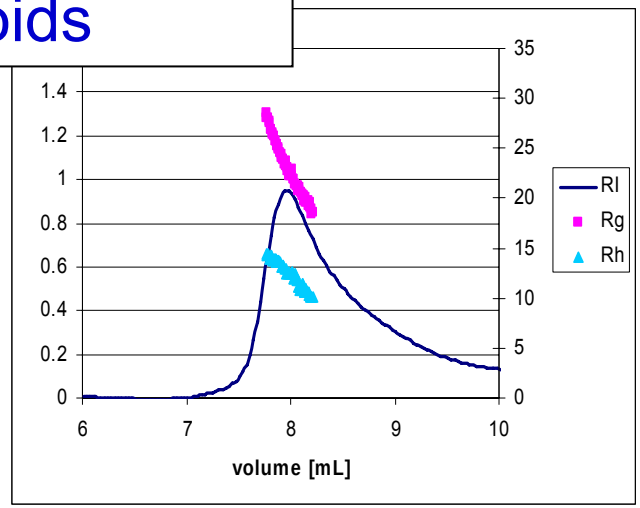


Combination of MALS (R_g) and DLS (R_h)

Ovalbumin



Amyloids

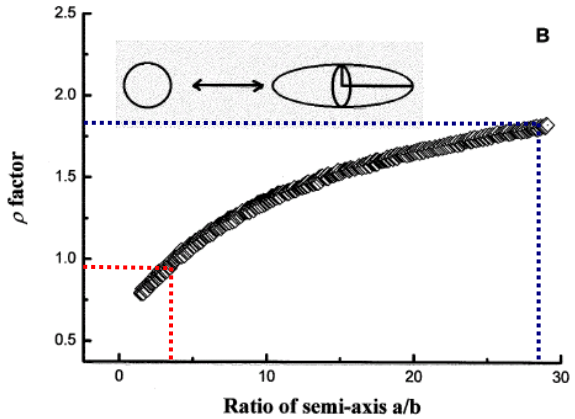


[B] Rizos, A. K., Spandidos, D. A., and Krambovitis, E., (2003) *Int. J. Med.,* 12, 559-563

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

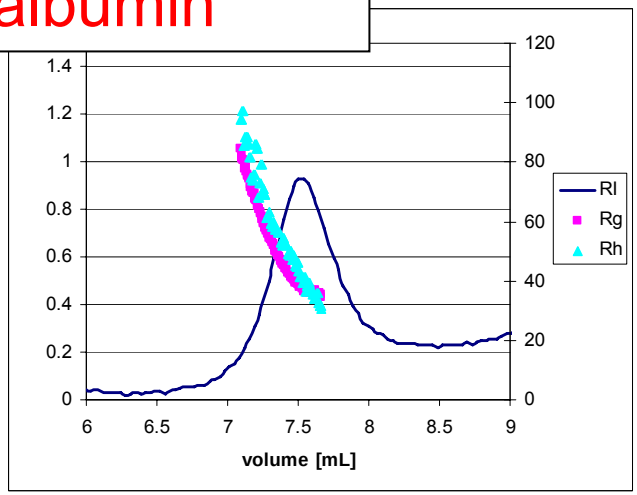
Aggregates of Ovalbumin *vs.* amyloid fibers

Shape factor: $\rho = R_g/R_h$



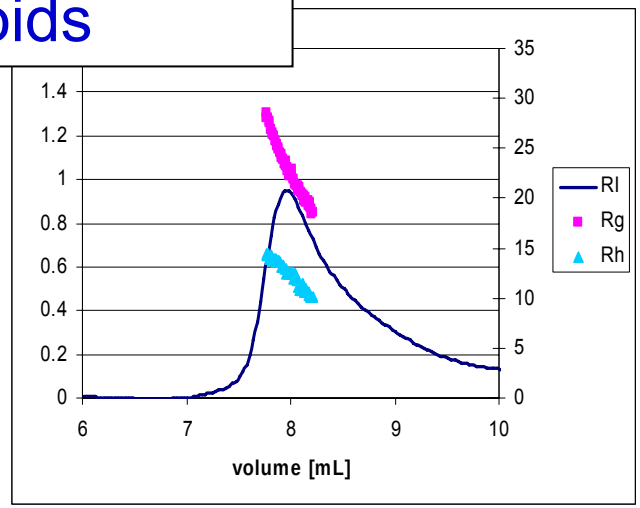
Combination of MALS (R_g) and DLS (R_h)

Ovalbumin



R_g/R_h = 0.91 Coil

Amyloids



R_g/R_h = 1.84 Rod

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

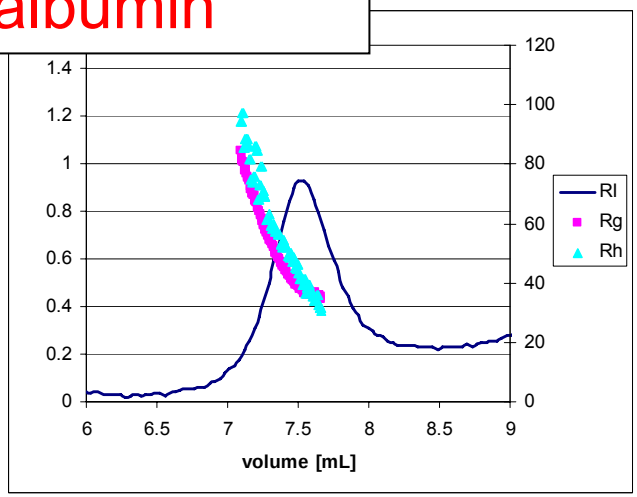
Aggregates of Ovalbumin *vs.* amyloid fibers

Shape factor: $\rho = R_g/R_h$

Combination of MALLS (R_g) and DLS (R_h)

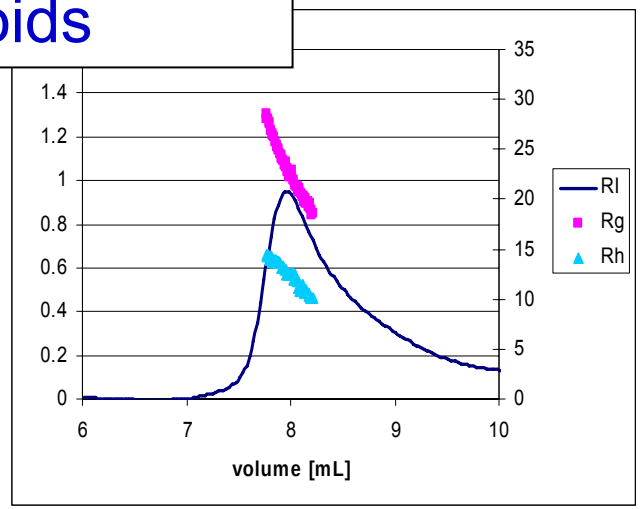
For	$\rho = R_g/R_h$
Sphere	0.774
Coil	0.816
Rod	1.732

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

Various uses of Light Scattering for assessing protein aggregates

Experiment	Detects Aggregates	Information about population (distribution)	Challenge in use	Sample dilution	Speed
DLS	Yes	No	Low	No	Fast
Micro-batch MALS	Yes	No	High	No	Medium
SEC/MALLS/DLS	Yes	Yes	Medium	Yes	Medium

Determination of the oligomeric state of modified proteins from SEC-LS/UV/RI analysis

1. Glycosylated proteins
2. Proteins conjugated with polyethylene glycol
3. Membrane protein present as a complex with lipids and detergents

Input:

- Polypeptide sequence
- *Chemical nature of the modifier*

Results:

- Oligomeric state of the polypeptide
- Extend of modification (grams of modifier /gram of polypeptide)

"three detector method"

Three Detector Method

$$MW_p = \frac{k*(LS)(UV)}{\varepsilon(RI)^2}$$

MW_p	Molecular Weight (polypeptide)
ε	extinction coefficient
LS	light scattering intensity
UV	absorbance (ε)
RI	refractive index change
k	calibration constant

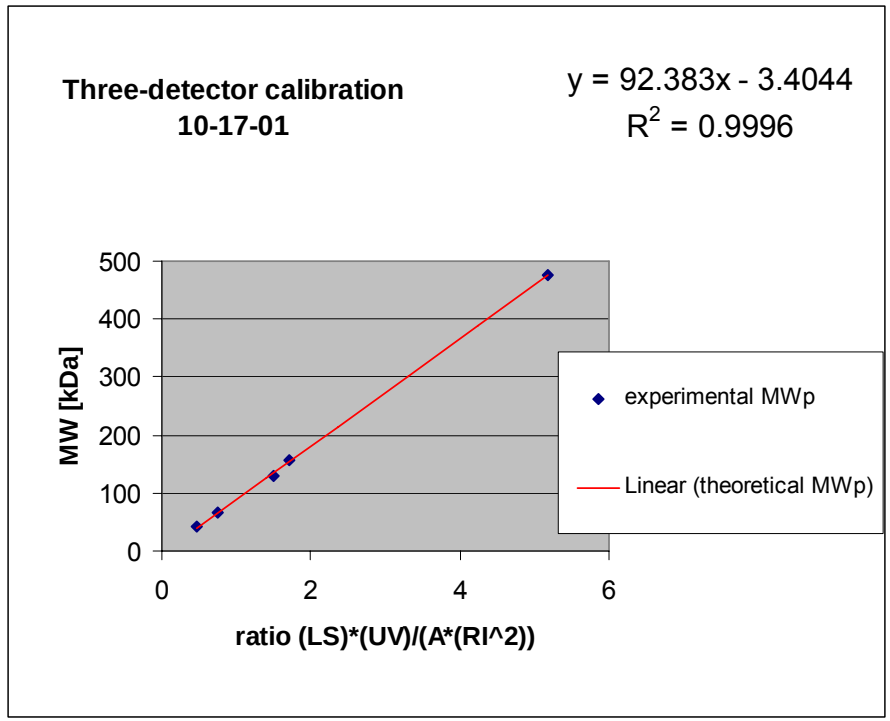
Yutaro Hayashi, Hideo Matsui and Toshio Takagi (1989) Methods Enzymol,172:514-28

Jie Wen, Tsutomu Arakawa and John S. Philo (1996) Anal Biochem, 240:155-66

Ewa Folta-Stogniew (2006) Methods in Molecular Biology: New and Emerging Proteomics Techniques, pp. 97–112

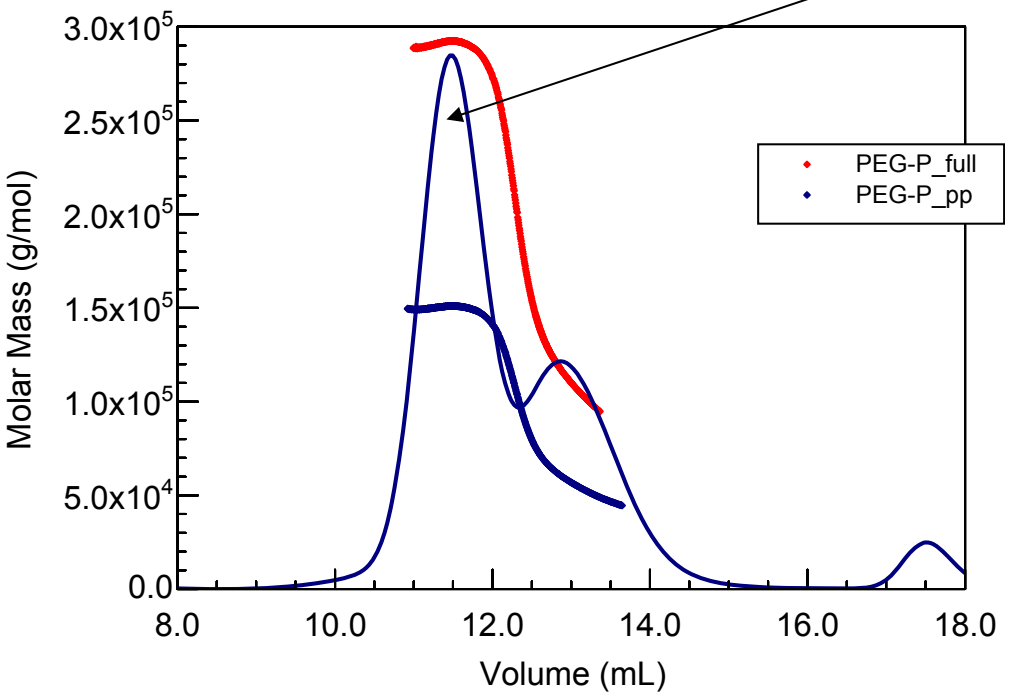
$$MW_p = \frac{k * (LS)(UV)}{\varepsilon (RI)^2}$$

Protein	MW (kDa)
Ova	43
BSA(1)	66
BSA(2)	132
Ald	156
Apo-Fer	475



PEG-ylated protein: 75 kDa

36 kDa polypeptide + 39 kDa PEG



Polypeptide: 146 kDa

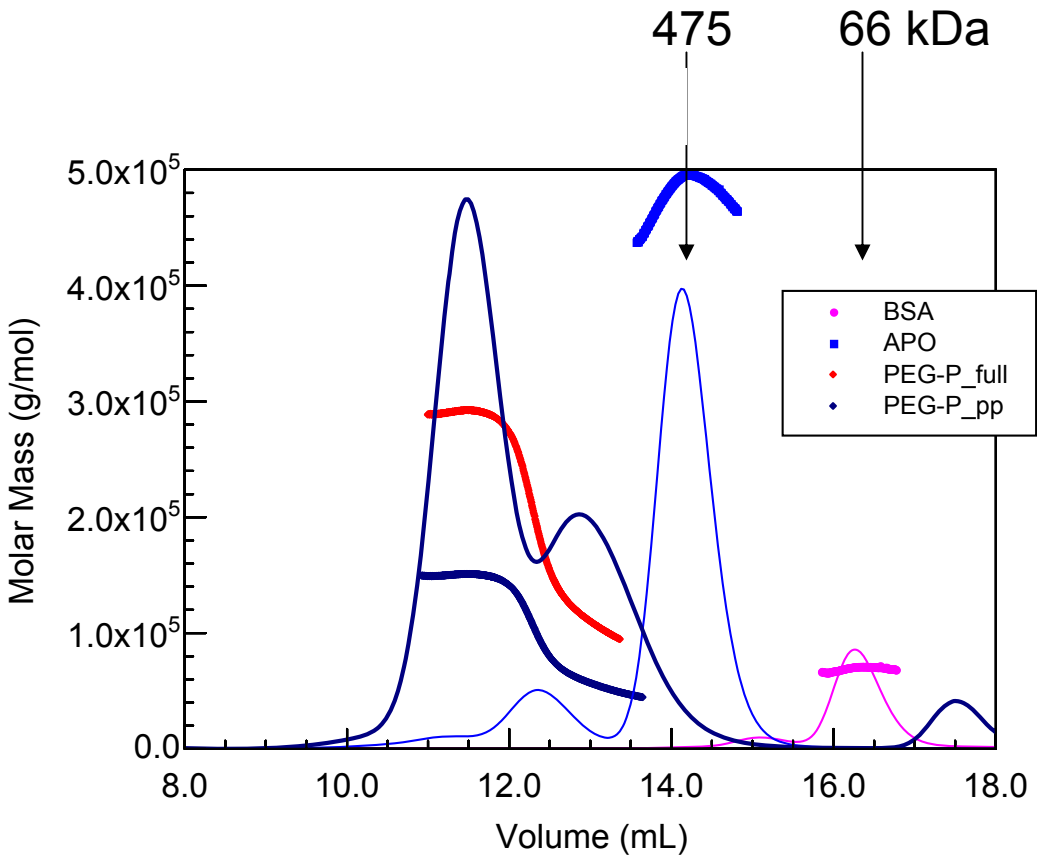
(tetramer: 144 kDa)

Full protein: 291 kDa

(tetramer: 300 kDa)

PEG-ylated protein: 75 kDa

36 kDa polypeptide + 39 kDa PEG



Polypeptide: 146 kDa

(tetramer: 144 kDa)

Full protein: 291 kDa

(tetramer: 300 kDa)

Capabilities

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 bias)
- SEC/MALS excellent in detecting and quantifying population with various oligomeric state in protein

Dynamic LS

- very fast detection of aggregates
- great dynamic range
- well suited to study kinetics of aggregation
- DLS detector available in a plate reader format for high volume analyses

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

- via frictional ratio Rh/Rs
- via shape factor ν, from log(Rg) vs. log(MM) plot
- via shape factor ρ, from Rg/Rh ratio

Limitations

Static LS

- measures weight average molar mass – needs fractionation to resolve different oligomeric states
- possible losses of sample during filtration and fractionation
- limitation on solvent choices (related to a fractionation step)
- SEC/SLS/DLS dilution during experiment

Dynamic LS

- measures hydrodynamic radius, which is affected by shape
- cannot discriminate between shape effects and changes in oligomeric states, *i.e.* non-spherical shape mimics oligomerization
- needs fractionation to resolve low number oligomers when present in mixture

Ken Williams
Director of W.M. Keck Biotechnology Resource
Laboratory at Yale University School of Medicine

NIH

Users of SEC/LS Service

http://info.med.yale.edu/wmkeck/biophysics

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