Determination of Molecular Masses of Proteins in Solution; Implementation of an HPLC Size Exclusion Chromatography and Laser Light Scattering Service in a Core Laboratory

Static and Dynamic LS

Experimental Set-Up

Parameters derived

Static LS

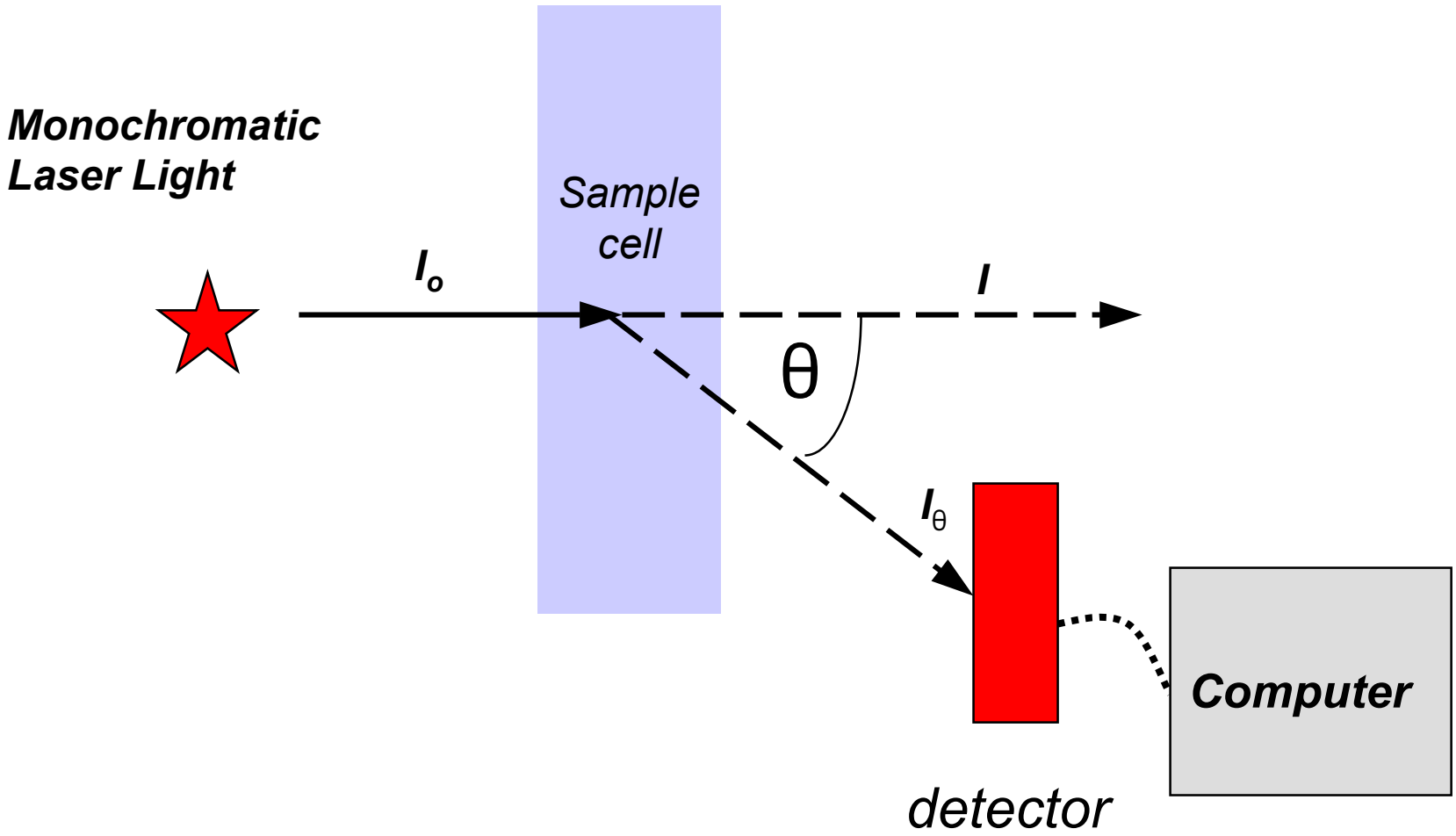
- Theory
- SEC/LS “in-line” Set Up
- Results for Standards
- Sample Requirements
- Applications

Dynamic LS

- Theory
- Results for Standards
- Batch mode *vs.* SEC/LS “in-line” measurements

Static vs. 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

Parameters derived:

- *MW (weight-average)*
- $(\langle r_g^2 \rangle^{1/2})$ root mean square radii for $(\langle r_g^2 \rangle^{1/2}) > (\lambda / 20) \sim 30\ nm$

Parameters derived:

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

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***

Static Light Scattering

- ***Theory***
- *SEC/LS “in-line” Set Up*
- *Results for Standards*
- *Sample Requirements*
- *Applications*

Static Light Scattering Experiments

Debye-Zimm formalism for R(θ), the excess intensity of scattered light at an angle θ

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

c	is the sample concentration (g/ml)
M_w	is the weight-average molecular weight (molar mass)
A_2	is the second virial coefficient (ml-mol/g^2)
K*	is an optical parameter equal to $4\pi^2n^2 (dn/dc)^2 /(\lambda_0{}^4N_A)$
n	is the solvent refractive index and dn/dc is the refractive index increment
N_A	is Avogadro's number
λ_0	is the wavelength of the scattered light in vacuum (cm)
$P(\theta)$	is the form factor (describes angular dependence of scattered light)

Static Light Scattering Experiments

$$\frac{K^*c}{R(\theta)} = \frac{1}{Mw}\left(1+\left(16\pi^2/3\lambda^2\right)<r_g{}^2>\sin^2\left(\frac{\theta}{2}\right)\right)$$

Using a multi angle instrument

construct a plot of

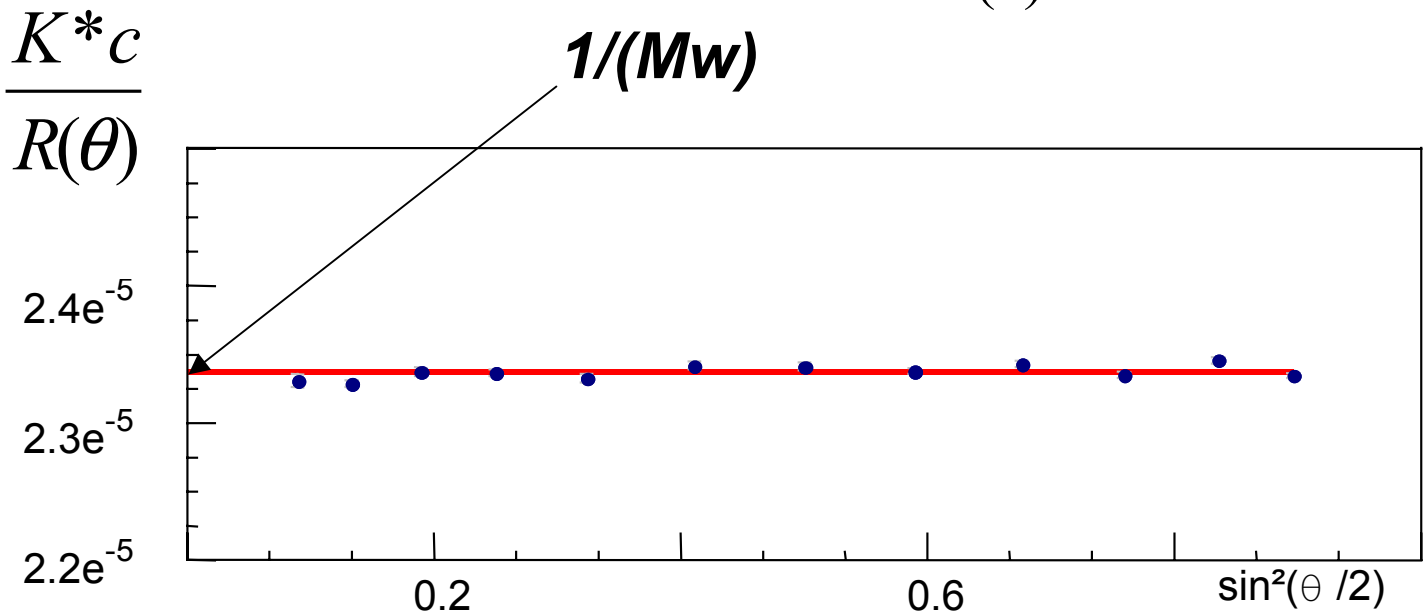
$$\frac{K^*c}{R(\theta)} \quad \textit{against} \quad \sin^2\left(\frac{\theta}{2}\right)$$

From intercept → Derived MW

weight-average

Zimm Plot Ovalbumin (43 kDa)

$$\frac{K^*c}{R(\theta)} = \frac{1}{Mw}\left(1 + f\left(\sin^2\left(\frac{\theta}{2}\right)\right)\right)$$

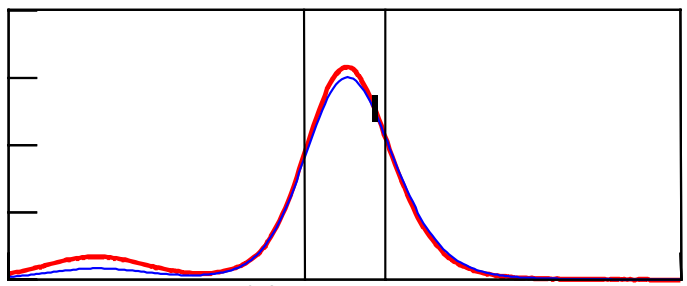


Volume : 16.300 mL

Conc. : (0.173 ± 0.000) mg/mL

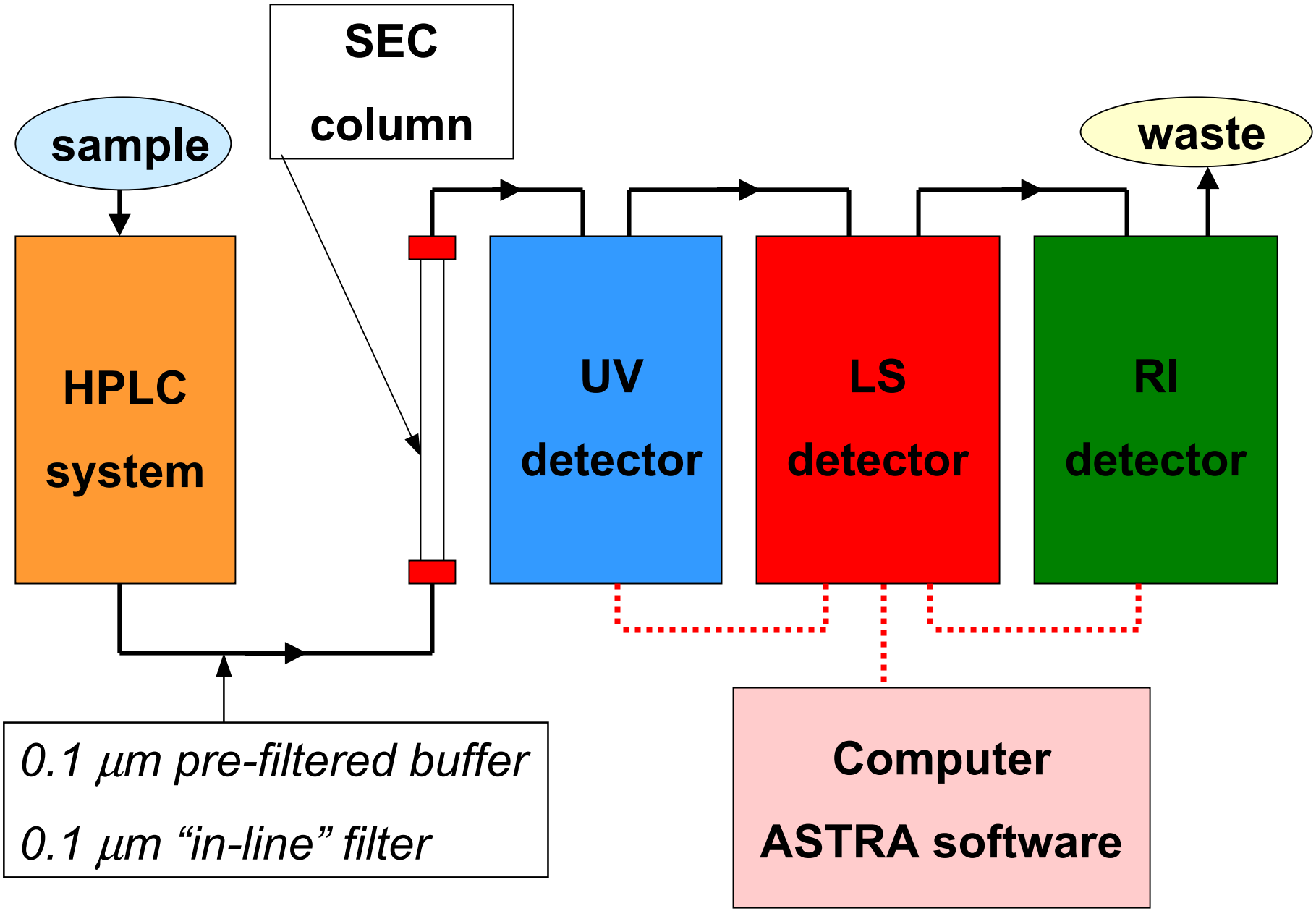
Mw (42.79 ± 0.03) x10^3 g/mol

Radius : 0.0 ± 0.0 nm



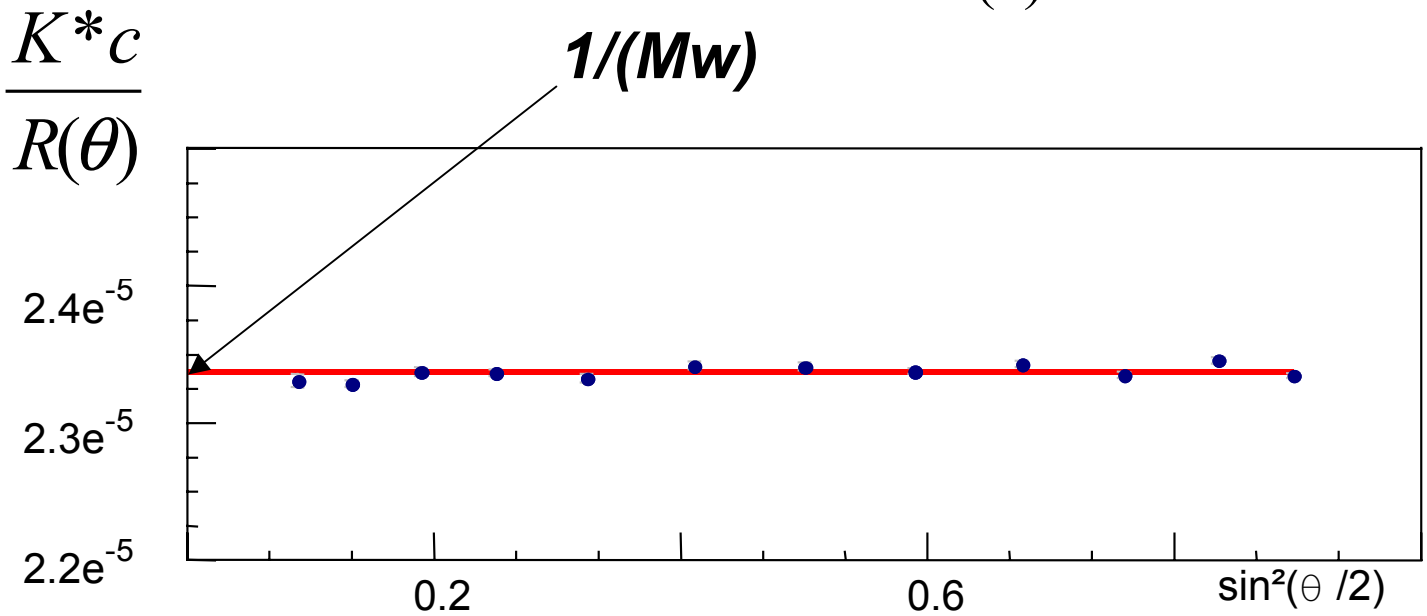
Static Light Scattering

- ***Theory***
- ***SEC/LS "in-line" Set Up***
- *Results for Standards*
- *Sample Requirements*
- *Applications*


SEC
column
sample
waste
HPLC
system
UV
detector
LS
detector
RI
detector
0.1 μm pre-filtered buffer
0.1 μm "in-line" filter
Computer
ASTRA software

Zimm Plot Ovalbumin (43 kDa)

$$\frac{K^*c}{R(\theta)} = \frac{1}{Mw}\left(1 + f\left(\sin^2\left(\frac{\theta}{2}\right)\right)\right)$$

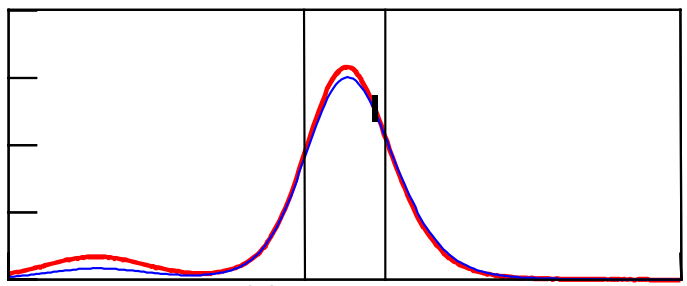


Volume : 16.300 mL

Conc. : (0.173 ± 0.000) mg/mL

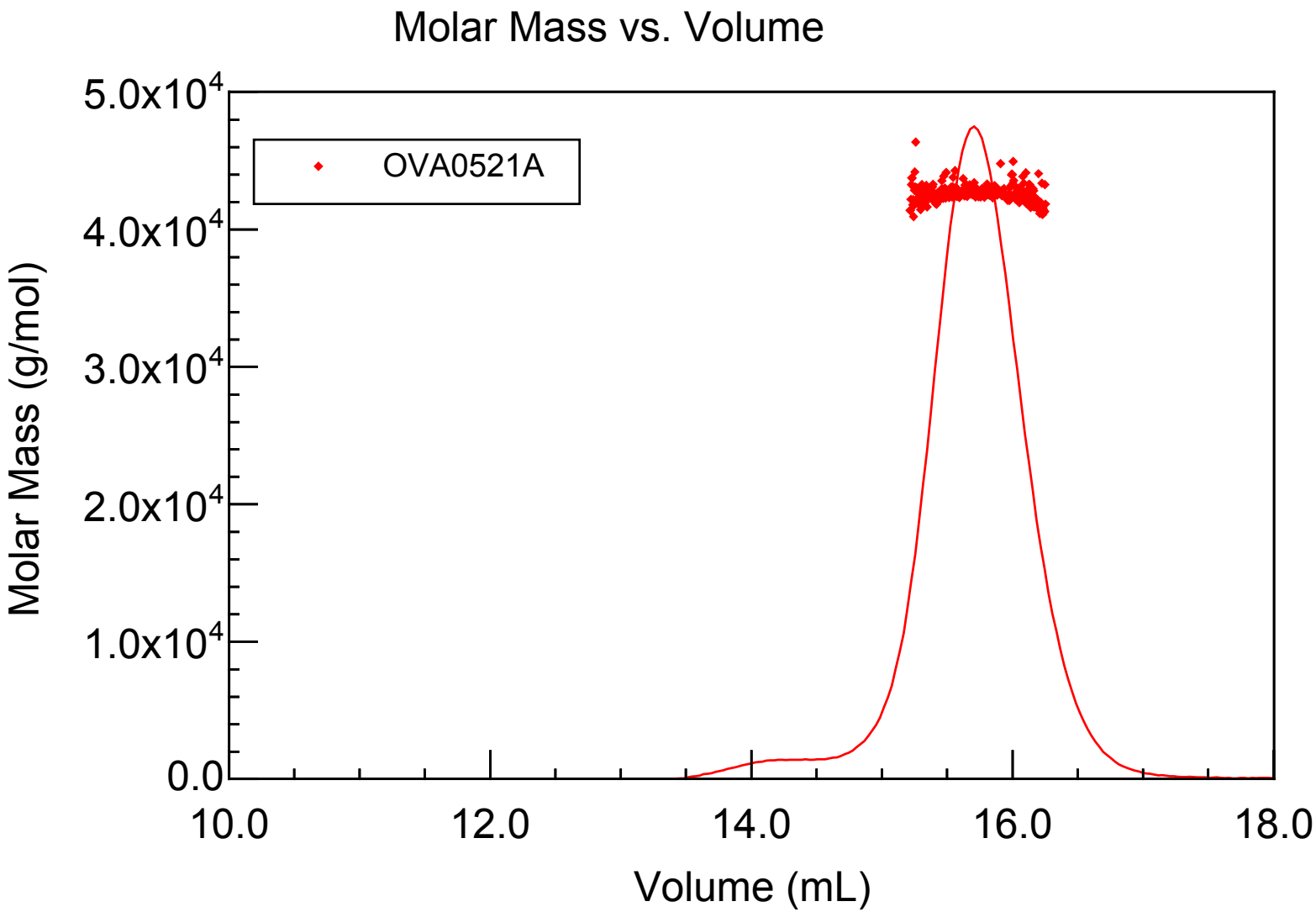
Mw (42.79 ± 0.03) x10^3 g/mol

Radius : 0.0 ± 0.0 nm



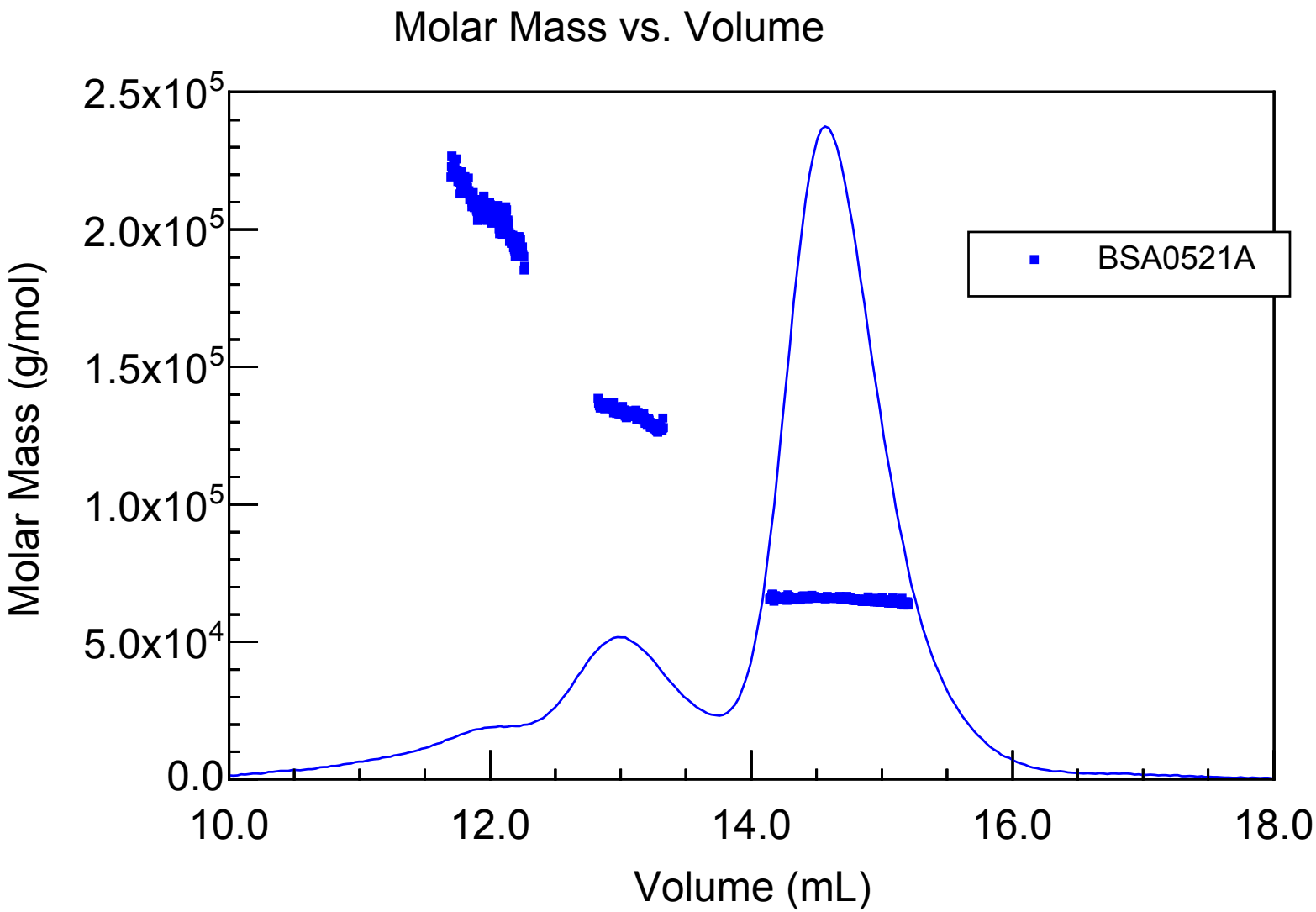
Molar Mass Distribution Plot

Ovalbumin 43 kDa



Molar Mass Distribution Plot

BSA 66 kDa



Static Light Scattering

- ***Theory***
- ***SEC/LS "in-line" Set Up***
- ***Results for Standards***
- *Sample Requirements*
- *Applications*

Molecular Weights Determined from "in line" analyses; static LS with SEC in line

Protein	Oligomeric state	# Runs	Pred. MW (kDa)[a]	Average MW ± St. Dev. (kDa)	Average error (%)
Aprotinin	monomer	2	6.5	6.8 ± 0.5	4.6
Cytochrome C	monomer	5	12.3	12.01 ± 0.57	2.4
α-Lactalbumin	monomer	2	14.2	14.32 ± 0.01	0.9
Myoglobin	monomer	3	17.0	14.19 ± 0.91	16
β-Lactglobulin	monomer	2	18.3	20.06 ± 0.33	9.7
Tripsin inhibitor	monomer	1	20.0	20.50	2.3
Carbonic anhydrase	monomer	4	29.0	29.22 ± 0.20	0.8
Ovalbumin	monomer	10	42.8	42.52 ± 0.68	1.4
BSA (monomer)	monomer	5	66.4	66.41 ± 1.00	1.2
Transferrin	monomer	2	75.2	76.92 ± 0.98	2.3
Enolase (yeast)	dimer	3	93.3	80.74 ± 1.18	13
Enolase (rabbit)	dimer	4	93.7	86.44 ± 1.90	7.8
BSA (dimer)	dimer	5	132.9	137.10 ± 3.93	3.2
Alc. dehydrogenase	tetramer	4	147.4	144.02 ± 0.86	2.4
Aldolase (rabbit)	tetramer	2	156.8	153.7 ± 1.91	1.1
Apo-ferritin	24 [x] monomer	2	475.9	470.3 ± 2.62	1.2
				Median error:	**2.3**

Buffer: 20 mM HEPES, 150 mM KCl, 1 mM EDTA, pH=8.0; column: Superdex 200 or Superdex 75

Correlation between the amount of protein analyzed and the accuracy of MW determination

Protein	Amount loaded (μg)	# Runs	Pred. MW (kDa)	Avrg. MW (kDa)	SD (kDa)	Avrg. error (%)	Range of accuracy (%)
Ovalbumin	150	4	42.8	42.4	0.3	0.9	0.2 to 1.6
	100	7	42.8	42.3	0.8	1.2	0.2 to 2.4
	45-50	4	42.8	41.6	1	2.8	0.5 to 5.8
	6-10	5	42.8	42.9	2	0.2	1.4 to 4.5
Transferrin	100	3	75.2	76.5	1	1.7	0.7 to 3.2
	8	5	75.2	76.3	2	1.5	0.3 to 5.2

column: TSK GEL $G3000_{SWXL}$ [TosoHaas], buffer: 20 mM phosphate, 150 mM NaCl, pH=7.5

Static Light Scattering

- ***Theory***
- ***SEC/LS "in-line" Set Up***
- ***Results for Standards***
- ***Sample Requirements***
- *Applications*

Sample requirements for proteins.

Column	Optimal amount of protein			Total volume of the eluting peak
	for expected MW >40 kDa	for expected MW 10 - 40 kDa	for expected MW<10 kDa	
Superose 6 (Pharmacia)	100 μg	N/A	N/A	~ 2mL
Superdex 200 (Pharmacia)	100 μg	200 - 300 μg	N/A	~ 2mL
Superdex 75 (Pharmacia)	50 μg	100 - 200 μg	400 μg	~ 1mL

Static Light Scattering

- *Theory*
- *SEC/LS “in-line” Set Up*
- *Results for Standards*
- *Sample Requirements*
- ***Applications***

SEC/LS Applications

- *Unusual elution positions*
- *Mixtures of non-interacting proteins*
- *Mixtures of interacting protein- detection of protein complexes*
- *Determination of the oligomeric state of mutant vs. wild type protein*

Please note the convention:

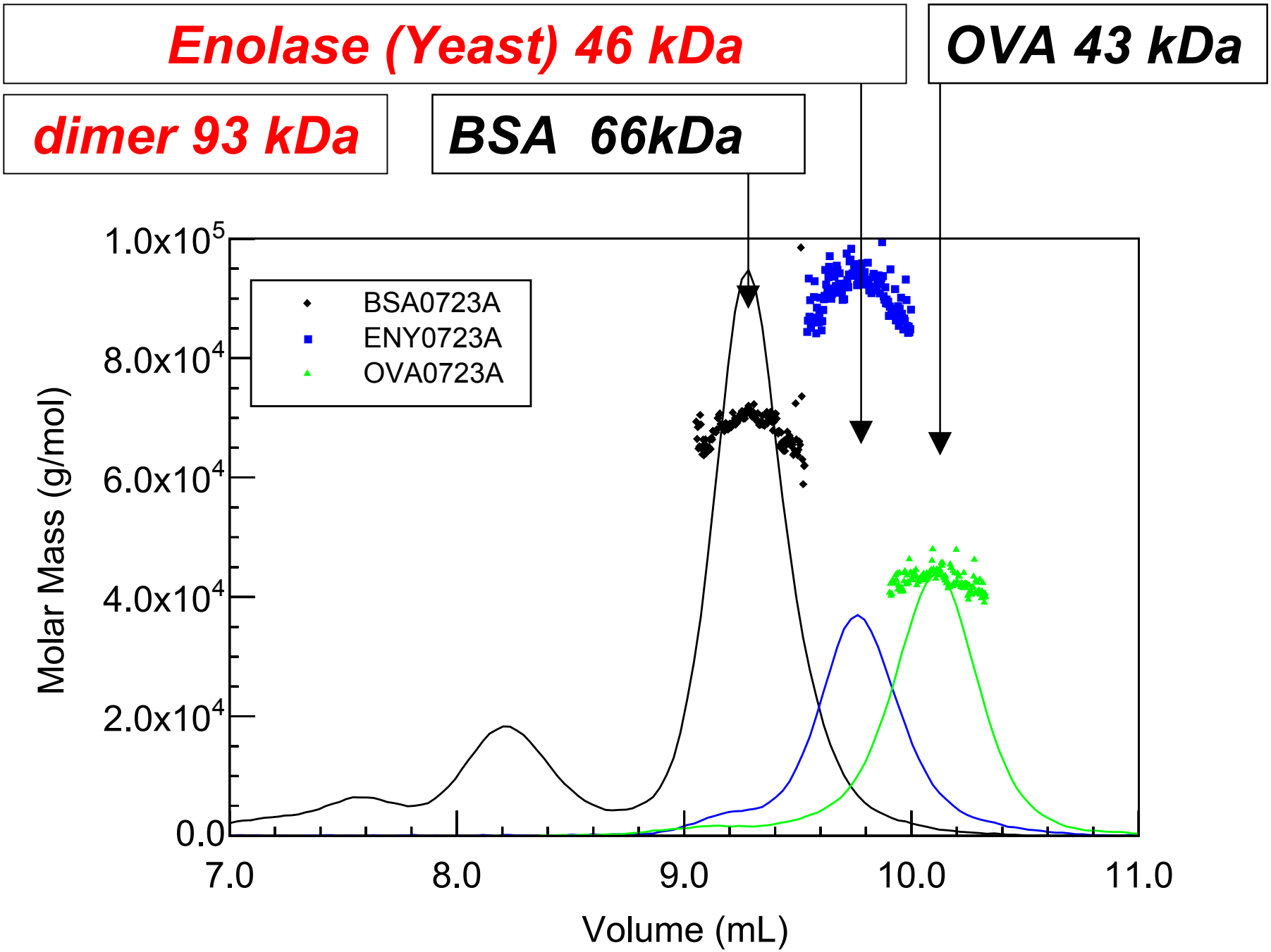
All the proteins are referred by MW of their monomeric forms

Unusual elution positions

Example:

BSA monomer - 66 kDa protein

Yeast Enolase - 93 kDa dimer (2x46kDa)


Enolase (Yeast) 46 kDa
OVA 43 kDa
dimer 93 kDa
BSA 66kDa
BSA0723A
ENY0723A
OVA0723A
Molar Mass (g/mol)
1.0x10^5
8.0x10^4
6.0x10^4
4.0x10^4
2.0x10^4
0.0
7.0
8.0
9.0
10.0
11.0
Volume (mL)

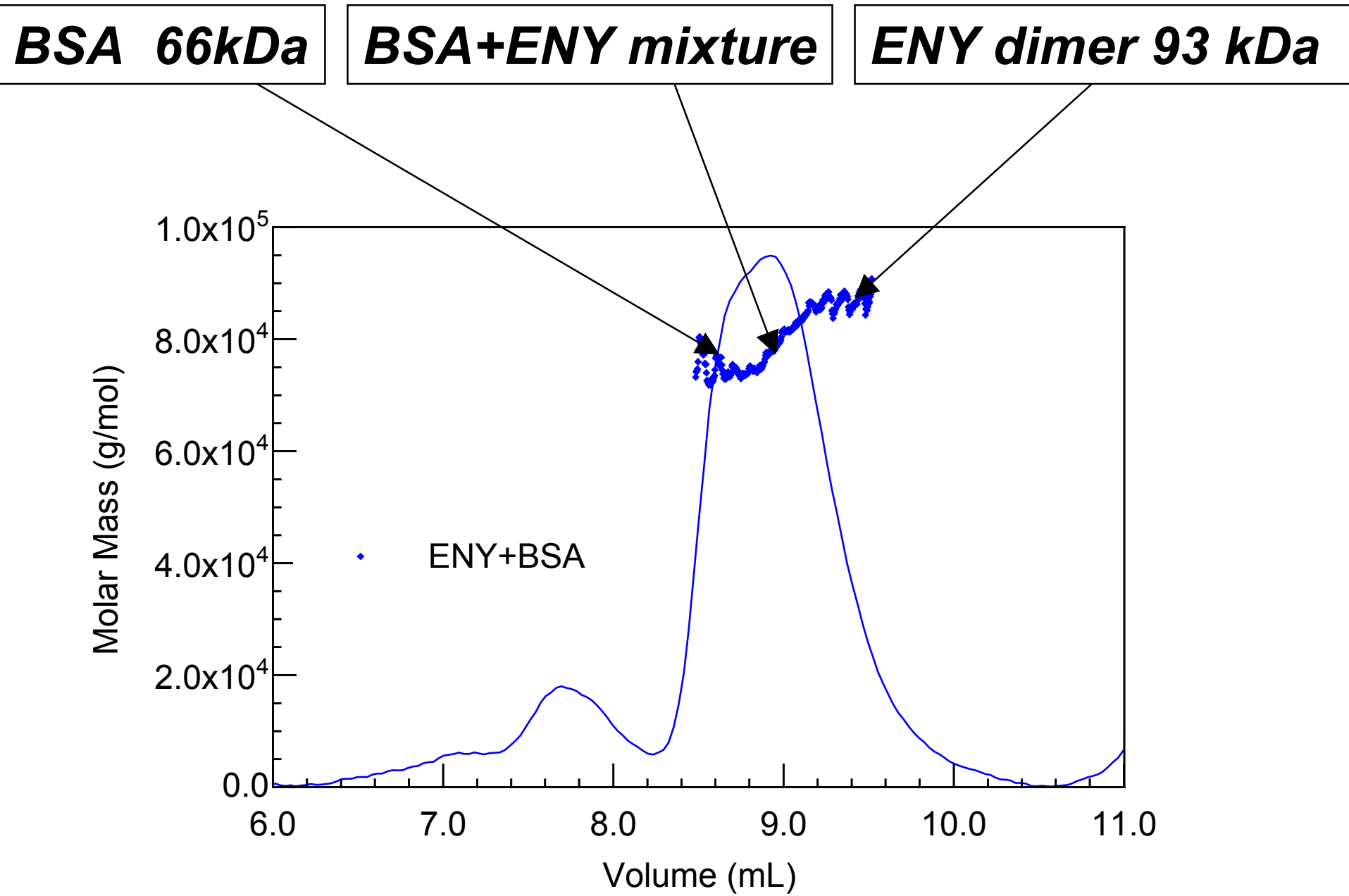
Mixtures of non-interacting proteins

Example:

BSA monomer - 66 kDa protein

Yeast Enolase - 93 kDa dimer (2x46kDa)

Analysis of co-eluting protein mixture



Analysis of interacting proteins

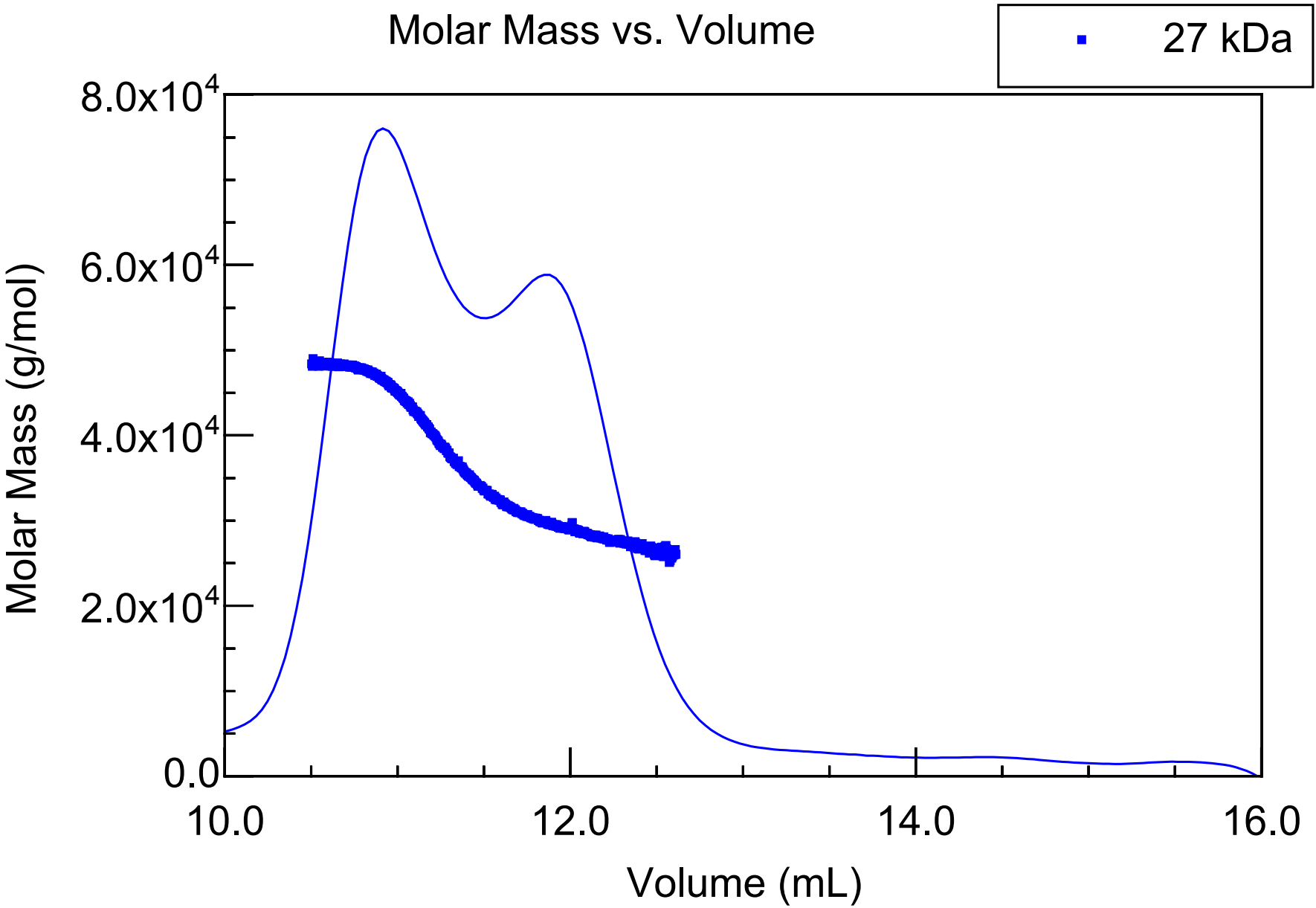
Example:

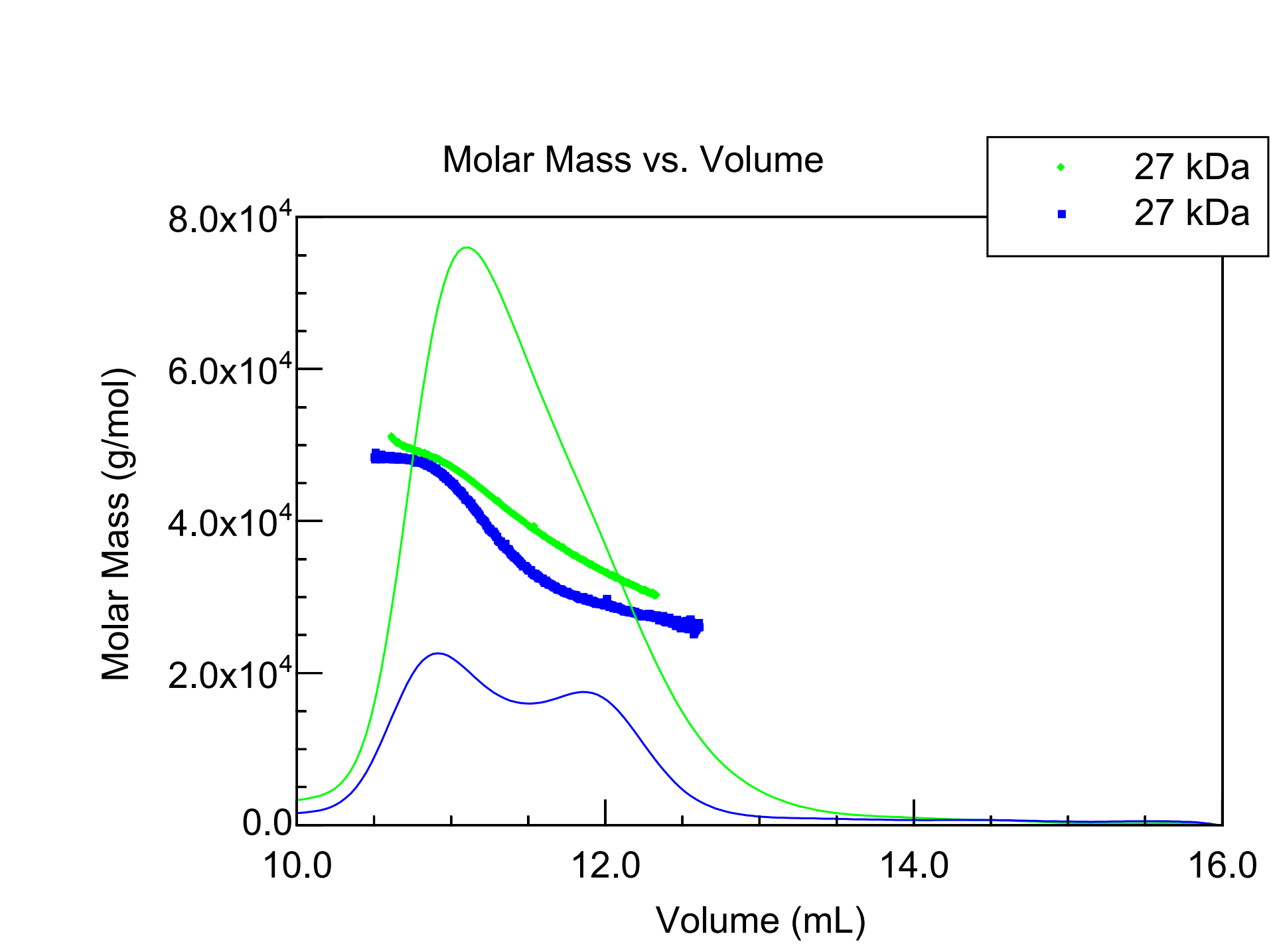
protein 27 kDa (protein exists as a mixture of monomer and dimer)

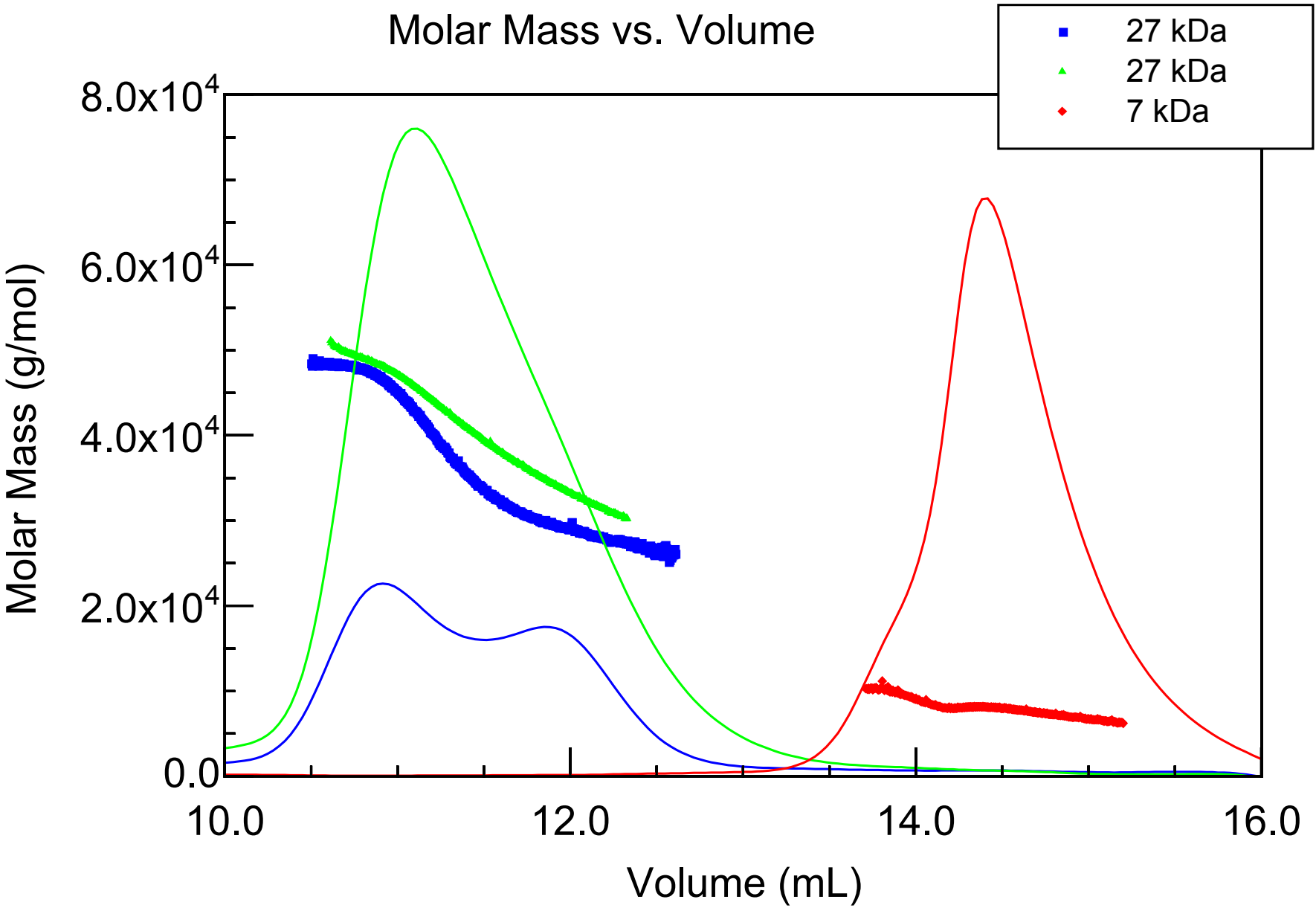
ligand 7 kDa

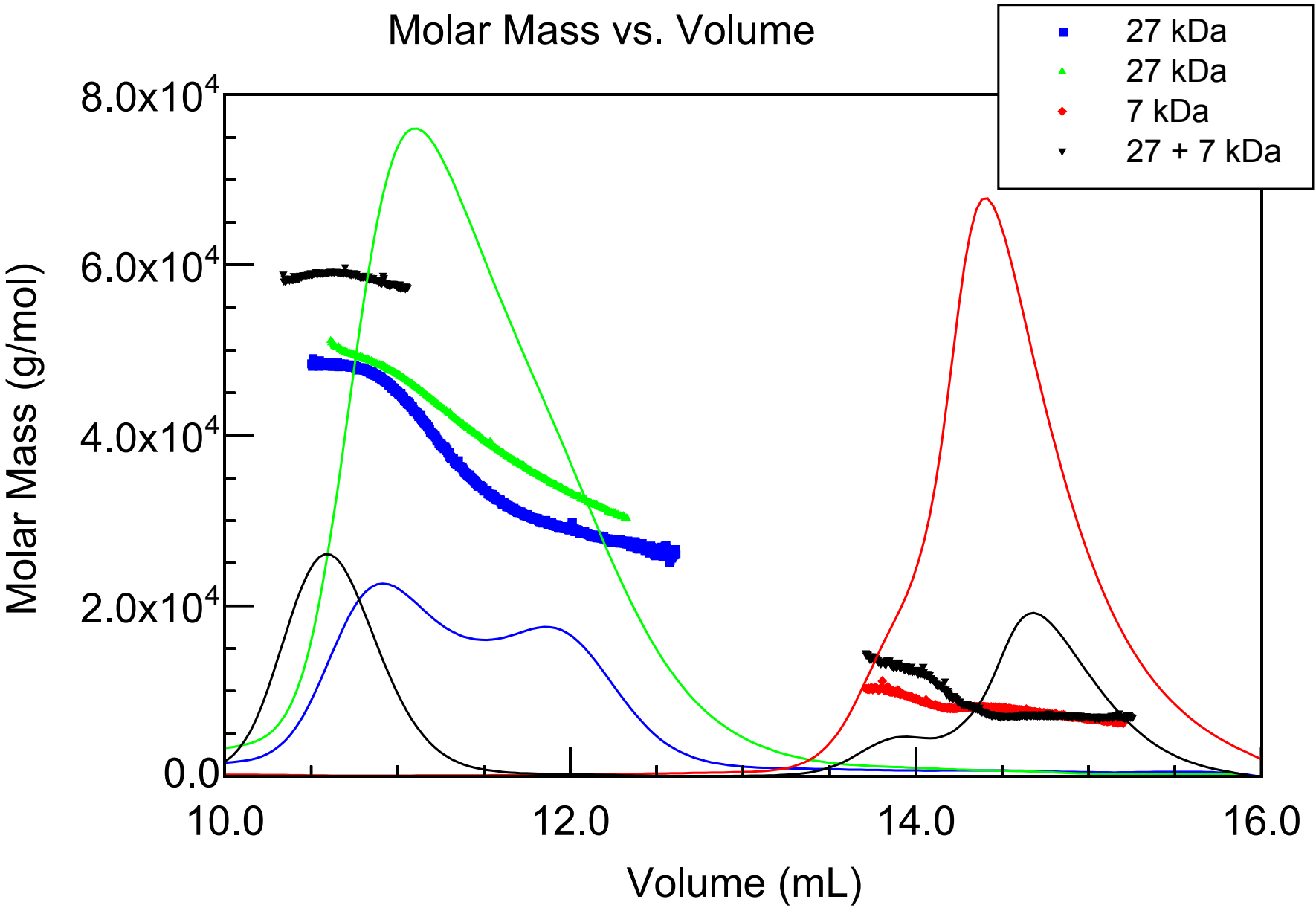
Ligand binding shifts the protein into dimeric form

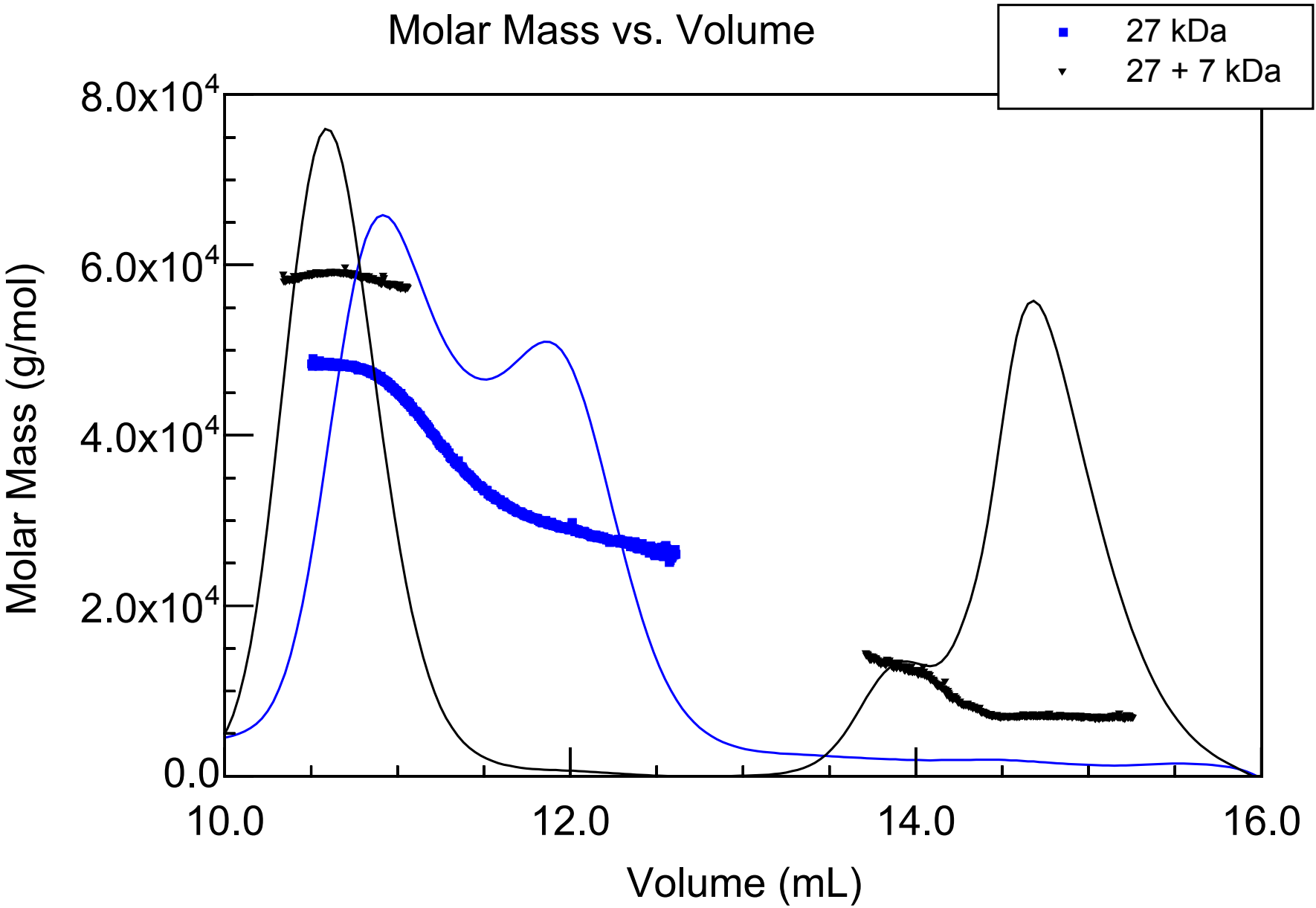
Analysis of interacting proteins




Molar Mass vs. Volume
27 kDa
27 kDa
8.0x10^4
6.0x10^4
4.0x10^4
2.0x10^4
0.0
Molar Mass (g/mol)
10.0
12.0
14.0
16.0
Volume (mL)


Molar Mass vs. Volume
27 kDa
27 kDa
7 kDa
8.0x10^4
6.0x10^4
4.0x10^4
2.0x10^4
0.0
Molar Mass (g/mol)
10.0
12.0
14.0
16.0
Volume (mL)


Molar Mass vs. Volume
27 kDa
27 kDa
7 kDa
27 + 7 kDa
8.0x10^4
6.0x10^4
4.0x10^4
2.0x10^4
0.0
Molar Mass (g/mol)
10.0
12.0
14.0
16.0
Volume (mL)


Molar Mass vs. Volume
27 kDa
27 + 7 kDa
8.0x10^4
6.0x10^4
4.0x10^4
2.0x10^4
0.0
Molar Mass (g/mol)
10.0
12.0
14.0
16.0
Volume (mL)

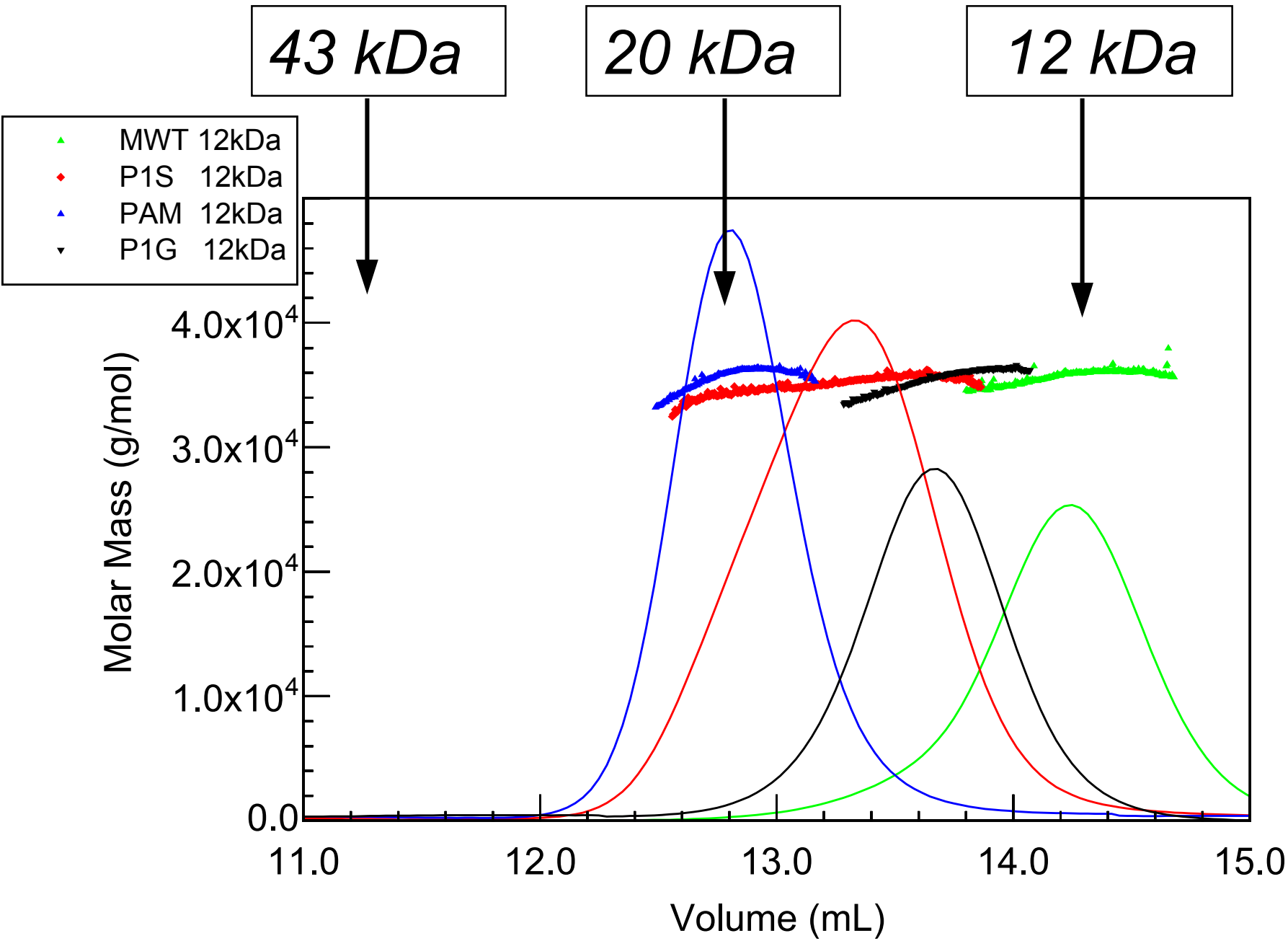
Determination of the oligomeric state of mutant vs. wild type protein

Example:

protein 12 kDa (WT protein exists as a trimer)

Three mutans and WT protein were analyzed.

There are significant differences in elution positions from SEC, however, all proteins were found to be trimeric forms- please note the abnormal elution position for each of the proteins.


43 kDa
20 kDa
12 kDa
MWT 12kDa
P1S 12kDa
PAM 12kDa
P1G 12kDa
Molar Mass (g/mol)
4.0x10^4
3.0x10^4
2.0x10^4
1.0x10^4
0.0
11.0
12.0
13.0
14.0
15.0
Volume (mL)

Dynamic Light Scattering

Light Scattering Experiments

- ***Static (classical)***

  time-averaged intensity of scattered light

- ***Dynamic (quasielastic)***

  fluctuation of intensity of scattered light with time

Parameters derived:

- *MW*
- $(\langle r_g^2 \rangle^{1/2})$ root mean square radii for $(\langle r_g^2 \rangle^{1/2}) > (\lambda / 20) \sim 30$ *nm*

Parameters derived:

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

Dynamic Light Scattering

- ***Theory***
- *Results for Standards*
- *Batch mode vs. SEC/LS “in-line” measurements*

Dynamic Light Scattering Experiments

fluctuation of scattered light intensity with time

comparison of scattering intensity at various time intervals (μsec) with the initial (t=0 sec) intensity

autocorrelator

constructing an autocorrelation function $g^{(2)}(\tau) = f(\tau)$

calculating the diffusion coefficient, D

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

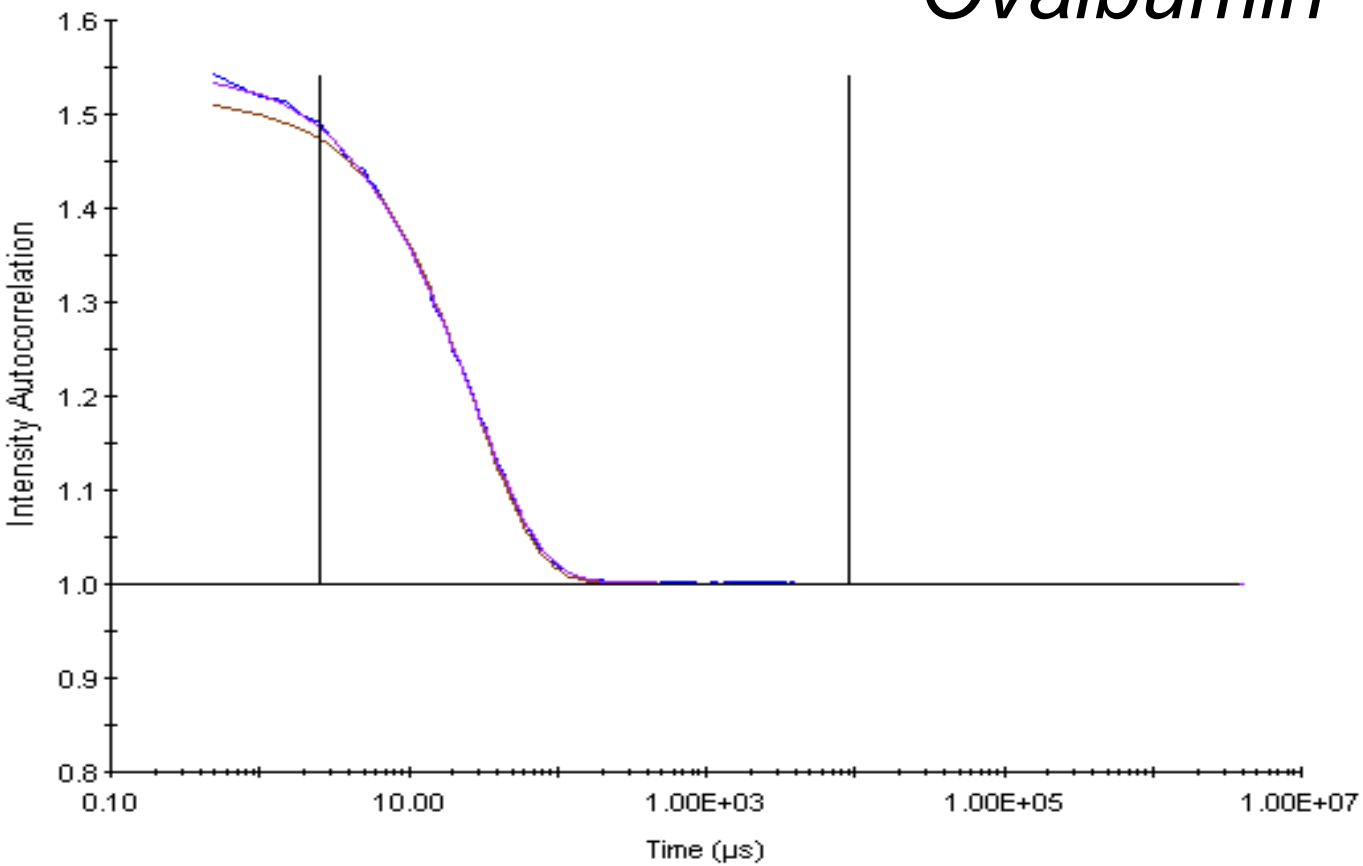
Stokes-Einstein equation

MODEL: dilute system of spherical molecules

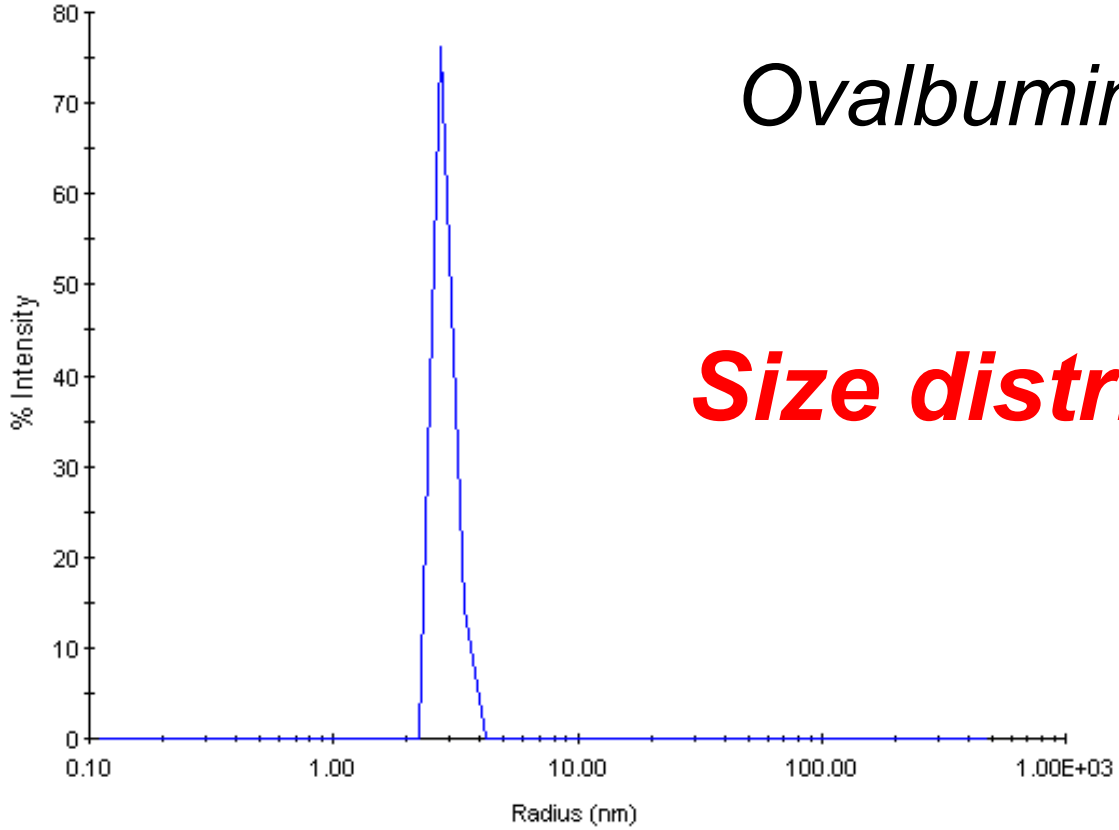
Dynamic Light Scattering Experiments

Autocorrelation function

Ovalbumin 43 kDa



Dynamic Light Scattering Experiments



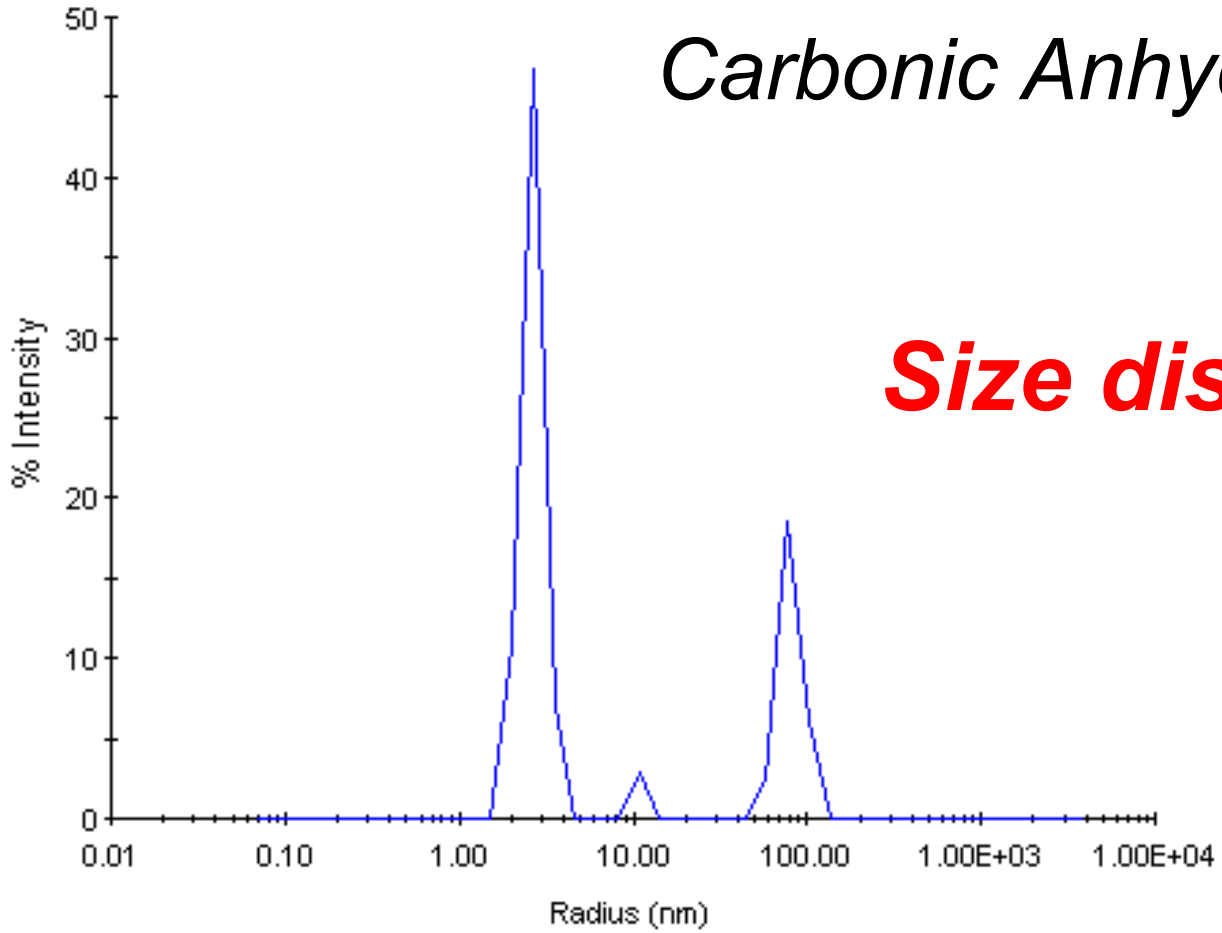
Ovalbumin 43 kDa

Size distribution

R=2.9 ± 0.2 nm *MW(R) = 40 kDa*

MW calculated from the calibration curve

Dynamic Light Scattering Experiments



Carbonic Anhydrase 29 kDa

Size distribution

R=2.7 ± 0.4 nm *MW(R) = 33 kDa*

Dynamic Light Scattering

- ***Theory***
- ***Results for Standards***
- *Batch mode vs. SEC/LS "in-line" measurements*

Hydrodynamic Radiuses and Molecular Weights Determined from DLS batch-mode analyses

Protein	Oligomeric state	# Runs	Radius ± SD (nm)	Average MW (kDa)	Predicted MW (kDa)	Avrg. error (%)
Aprotinin	monomer	15	1.64 ± .02	10.7	6.5	65
Cytochrome C	monomer	20	1.97 ± .05	16.6	12.3	35
α-Lactalbumin	monomer	25	2.09 ± .07	19.1	14.2	34
Myoglobin	monomer	25	2.27 ± .04	23.0	17.0	35
β-Lactglobulin	monomer	20	2.85 ± .05	38.8	18.3	111
Trypsin inhibitor	monomer	20	2.53 ± .05	29.4	20.0	47
Carbonic anhydrase	monomer	20	2.70 ± .03	34.7	29.0	19
Ovalbumin	monomer	30	3.21 ± .06	51.7	42.8	20
BSA (monomer)	monomer	20	3.97 ± .06	85.3	66.4	28
Transferrin	monomer	30	4.04 ± .13	88.5	75.2	18
Enolase (yeast)	dimer	25	3.78 ± .04	75.4	93.3	19
Alc. dehydrogenase	tetramer	20	4.52 ± .29	116.2	147.4	21
Aldolase (rabbit)	tetramer	25	5.70 ± .69	217.9	156.8	39
Apo-ferritin	24 ^{x} monomer	25	7.86 ± .21	420.4	475.9	12
					Median:	**31**

Results obtained in "batch-mode" for polydisperse samples

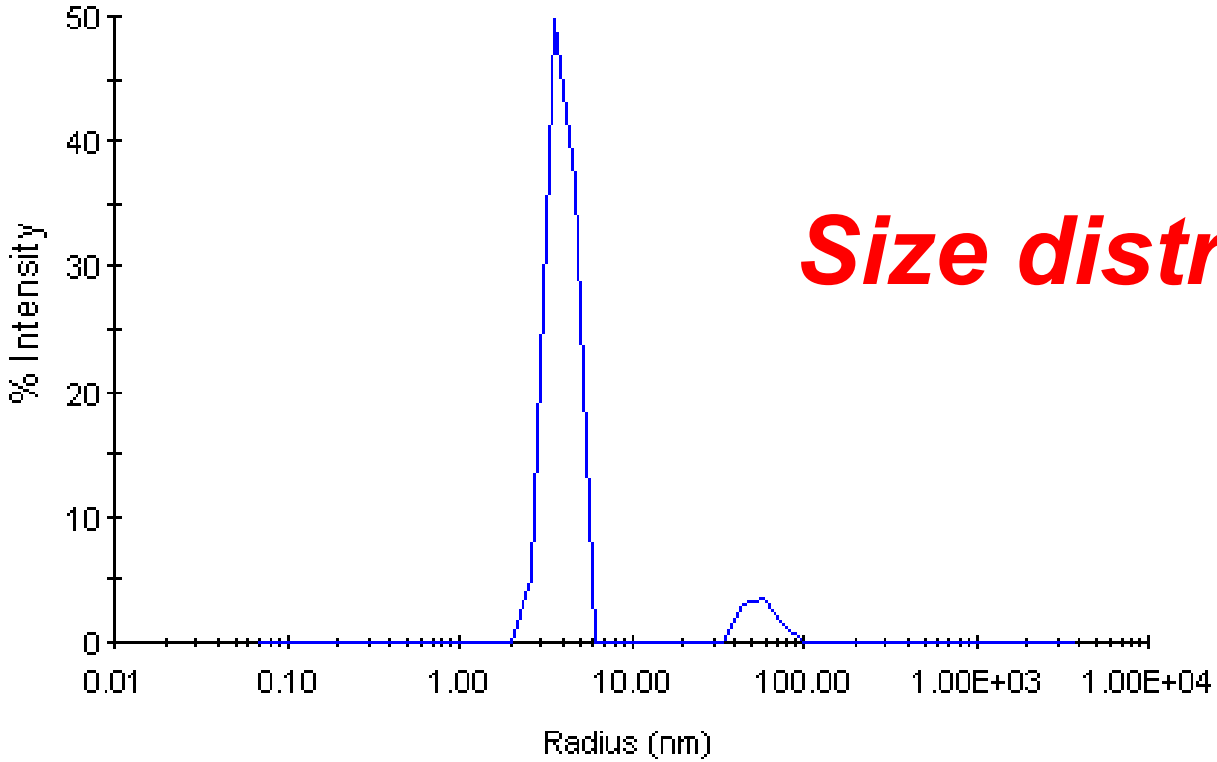
In "batch-mode" the DLS experiment is able to detect that the sample is POLYDISPERSE (i.e. the sample is not homogeneous in respect to oligomeric state); it cannot however discriminate what oligomeric form are present

Example:

BSA : mixture of monomer, dimers

Dynamic Light Scattering Experiments

BSA 66 kDa

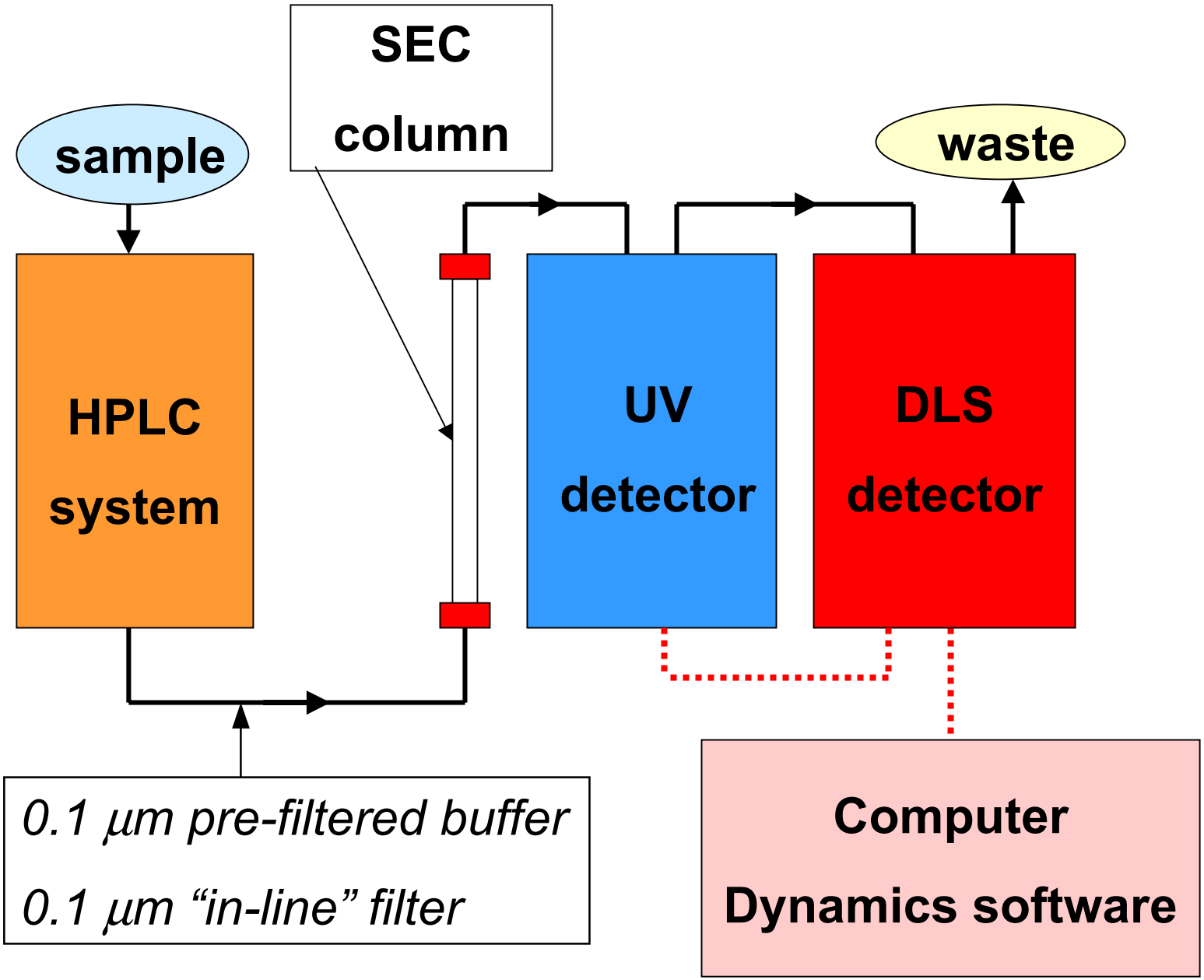


Size distribution

R=4.0 ± 0.6 nm *MW(R) = 84 kDa*

Dynamic Light Scattering

- ***Theory***
- ***Results for Standards***
- ***Batch mode vs. SEC/LS "in-line" measurements***


SEC
column
sample
waste
HPLC
system
UV
detector
DLS
detector
0.1 μm pre-filtered buffer
0.1 μm "in-line" filter
Computer
Dynamics software

Results obtained in "SEC/LS" mode for polydisperse samples

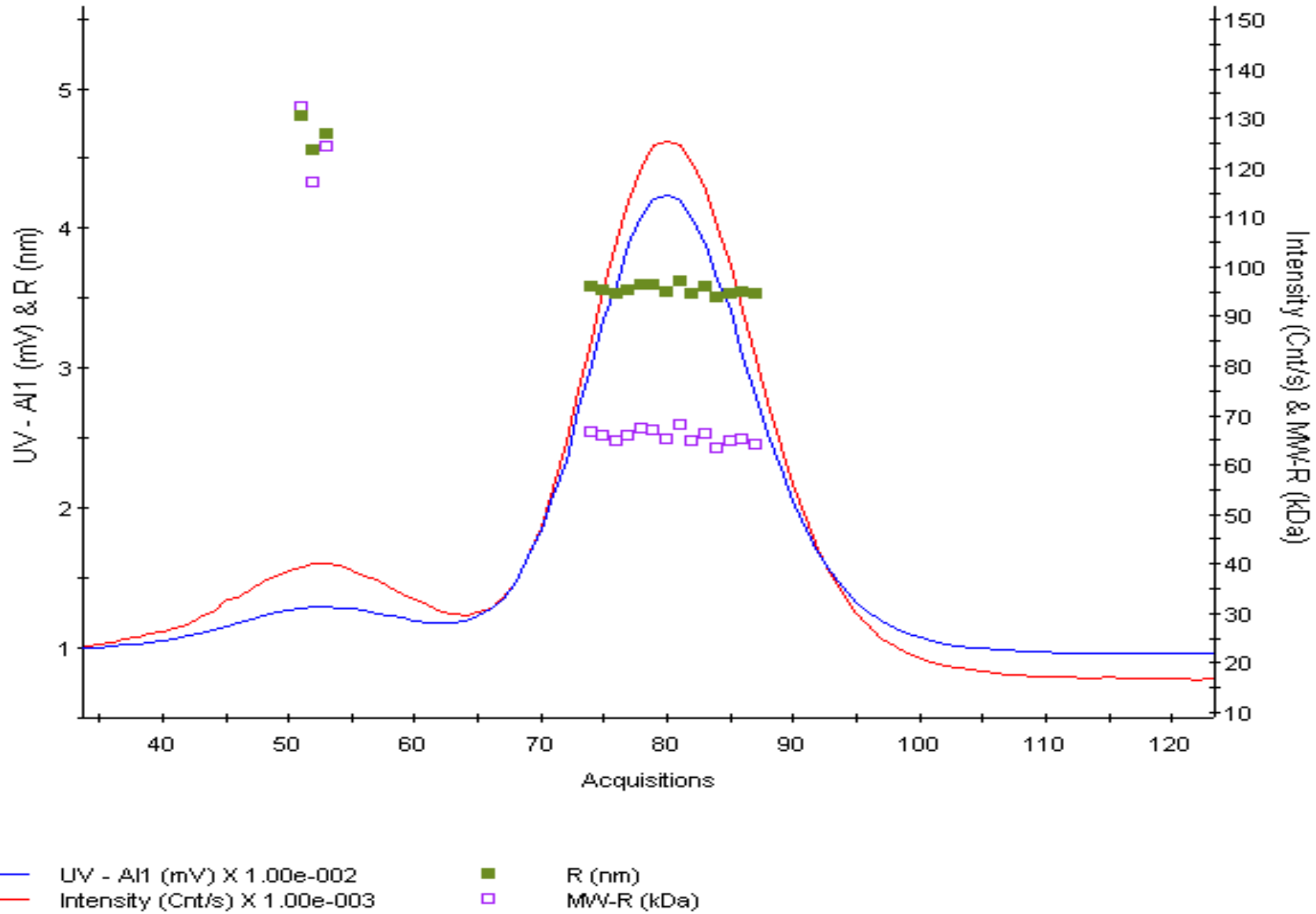
In "SEC/LS" mode, the SEC serves as a fractionation step enabling determination of oligomeric state for each of the oligomeric forms that are present in the sample

Example:

BSA : mixture of monomer, dimers

Molar Mass Distribution Plot

BSA 66 kDa



Results obtained in "SEC/LS" mode for standard proteins; data are reported for the major eluting peak

Hydrodynamic Radiuses and Molecular Weights Determined from "in-line" DLS analysis

Protein	Oligomeric state	# Runs	Radius ± SD (nm)	Average MW (kDa)	Predicted MW (kDa)	Average error (%)
Aprotinin	monomer	3	1.35 ± .06	6.8	6.5	4.9
Cytochrome C	monomer	3	1.77 ± .12	12.8	12.3	4.3
α-Lactalbumin	monomer	3	1.91 ± .08	15.3	14.2	7.8
Myoglobin	monomer	3	2.12 ± .07	19.5	17.0	14.4
β-Lactglobulin	monomer	3	2.64 ± .13	32.7	18.3	78.8
Trypsin inhibitor	monomer	3	2.47 ± .08	28.0	20.0	40.0
Carbonic anhydrase	monomer	3	2.35 ± .16	25.0	29.0	14.0
Ovalbumin	monomer	3	2.98 ± .02	43.5	42.8	1.6
BSA (monomer)	monomer	3	3.56 ± .01	65.8	66.4	0.9
Transferrin	monomer	3	4.02 ± .06	87.1	75.2	15.9
Enolase (yeast)	dimer	3	3.57 ± .02	66.0	93.3	29.3
Enolase (rabbit)	dimer	3	3.65 ± .10	69.7	93.7	25.6
BSA (dimer)	dimer	3	4.68 ± .21	125.1	132.9	5.9
Alc. dehydrogenase	tetramer	3	4.50 ± .10	113.8	147.4	22.8
Aldolase (rabbit)	tetramer	3	4.77 ± .06	130.5	156.8	16.8
					Median:	**20.0**

Hydrodynamic Radiuses and Molecular Weights Determined from "in-line" DLS analysis

Protein	Oligomeric state	# Runs	Radius ± SD (nm)	Average MW (kDa)	Predicted MW (kDa)	Average error (%)
Aprotinin	monomer	3	1.35 ± .06	6.8	6.5	4.9
Cytochrome C	monomer	3	1.77 ± .12	12.8	12.3	4.3
α-Lactalbumin	monomer	3	1.91 ± .08	15.3	14.2	7.8
Myoglobin	monomer	3	2.12 ± .07	19.5	17.0	14.4
β-Lactglobulin	monomer	3	2.64 ± .13	32.7	18.3	
Trypsin inhibitor	monomer	3	2.47 ± .08	28.0	20.0	40.0
Carbonic anhydrase	monomer	3	2.35 ± .16	25.0	29.0	14.0
Ovalbumin	monomer	3	2.98 ± .02	43.5	42.8	1.6
BSA (monomer)	monomer	3	3.56 ± .01	65.8	66.4	0.9
Transferrin	monomer	3	4.02 ± .06	87.1	75.2	15.9
Enolase (yeast)	dimer	3	3.57 ± .02	66.0	93.3	
Enolase (rabbit)	dimer	3	3.65 ± .10	69.7	93.7	
BSA (dimer)	dimer	3	4.68 ± .21	125.1	132.9	5.9
Alc. dehydrogenase	tetramer	3	4.50 ± .10	113.8	147.4	22.8
Aldolase (rabbit)	tetramer	3	4.77 ± .06	130.5	156.8	16.8
					Median:	**10.9**

Conclusions

Static LS

- fast and accurate determination of molecular weight (MW) of macromolecules in solution
- single SEC/LS measurement should be sufficient to estimate a MW with a precession of ± 5%
- SEC/LS suitable for characterization of non-interacting and interacting systems

Dynamic LS

- in batch mode, very fast evaluation of sample polydispersity
- fast and accurate determination of hydrodynamic radius in solution
- MW can be estimated (with a precession of ~10-20% for SEC/LS set-up)

Ken Williams

Director of HHMI Biopolymer & W.M. Keck Biotechnology Resource Laboratory

NIH

Thomas Mozdzer

Users of SEC/LS Service

Wyatt Technology *Protein Solutions*