

***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***

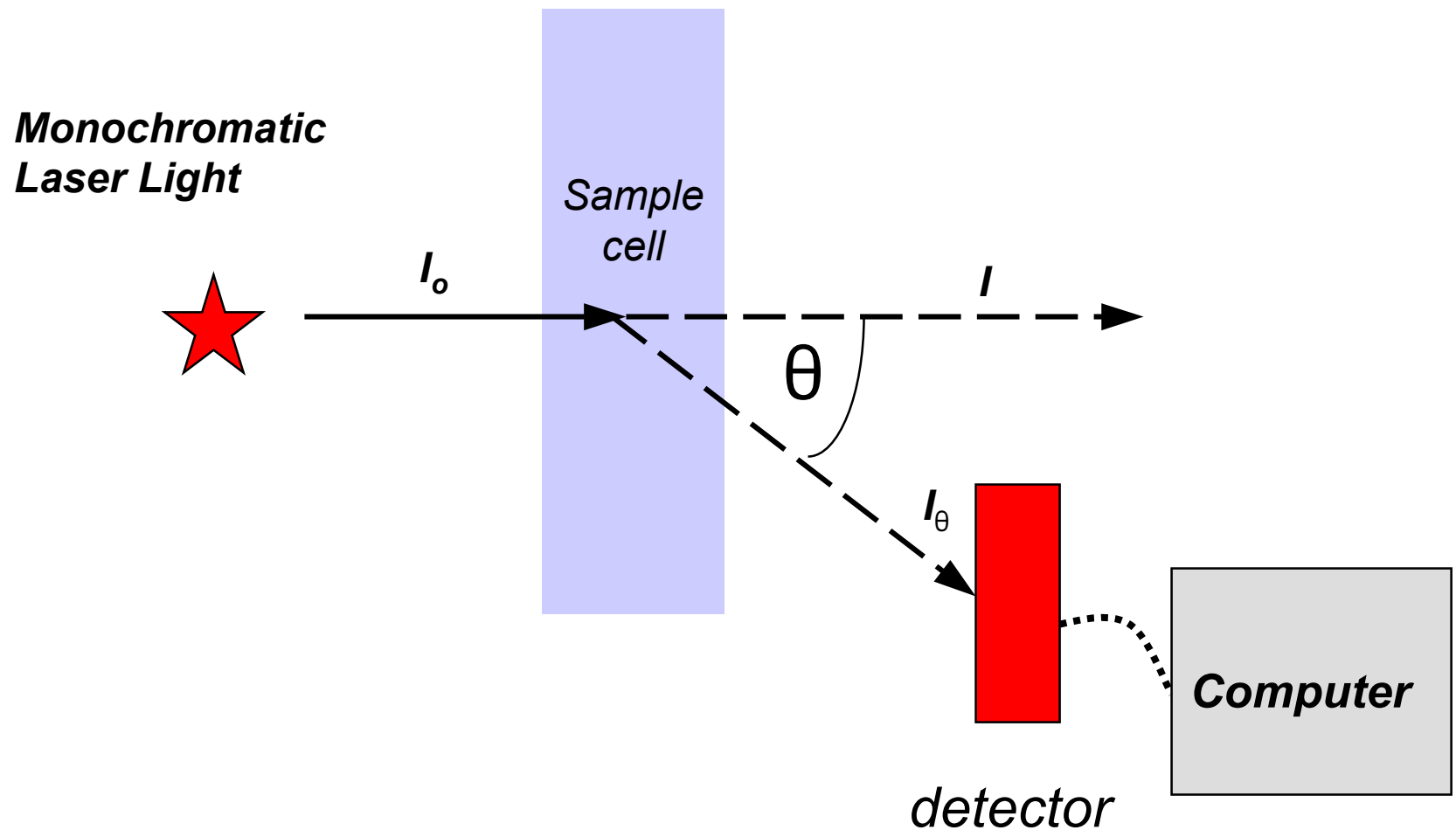
- Theory
- SEC/LS “in-line” Set Up
- Results for Standards
- Sample Requirements
- Applications

## ***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  $(\langle r_g^2 \rangle^{1/2}) > (\lambda/20) \sim 30 \text{ nm}$

## *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  $R(\theta)$ , the excess intensity of scattered light at an angle  $\theta$

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

- $c$  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<sup>2</sup>)
- $K^*$  is an optical parameter equal to  $4\pi^2 n^2 (dn/dc)^2 / (\lambda_0^4 N_A)$
- $n$  is the solvent refractive index and  $dn/dc$  is the refractive index increment
- $N_A$  is Avogadro's number
- $\lambda_0$  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

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

Using a multi angle instrument  
construct a plot of

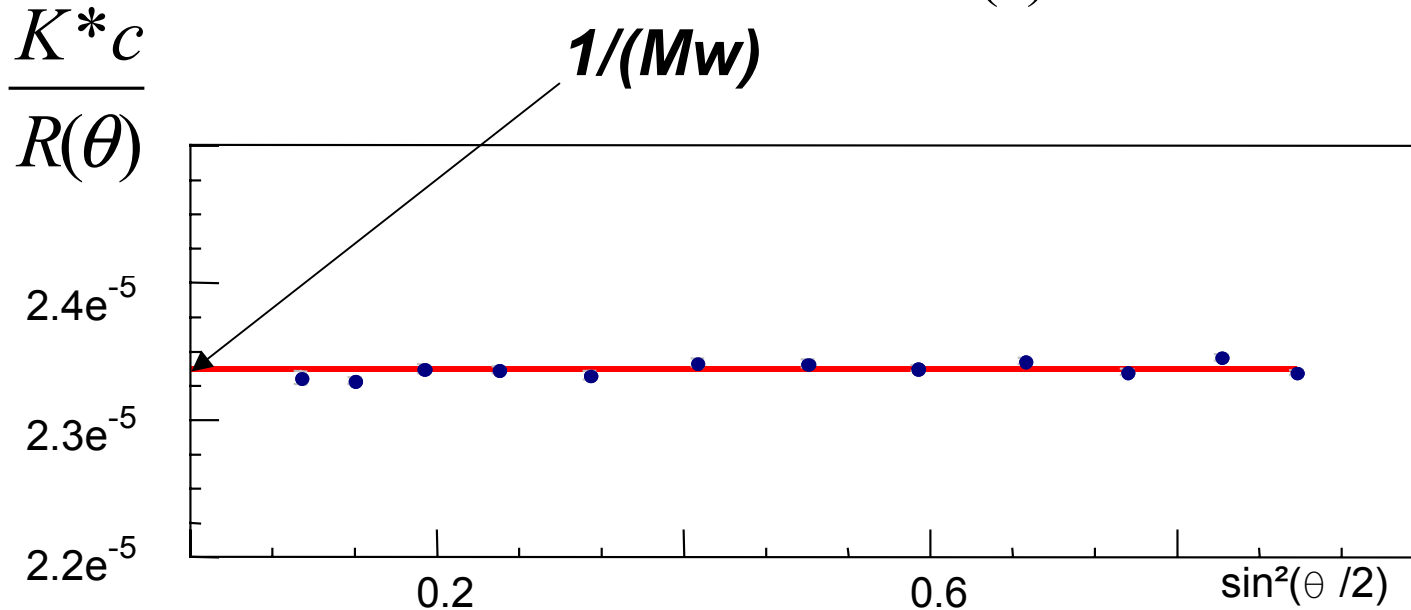
$$\frac{K^*c}{R(\theta)} \quad \text{against} \quad \sin^2\left(\frac{\theta}{2}\right)$$

**From intercept**  $\rightarrow$  **Derived MW**

*weight-average*

# Zimm Plot Ovalbumin (43 kDa)

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

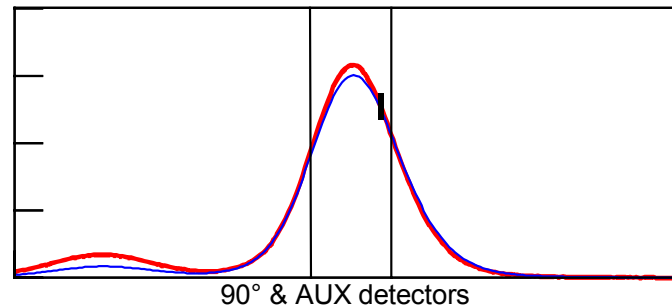


Volume : 16.300 mL

Conc. :  $(0.173 \pm 0.000)$  mg/mL

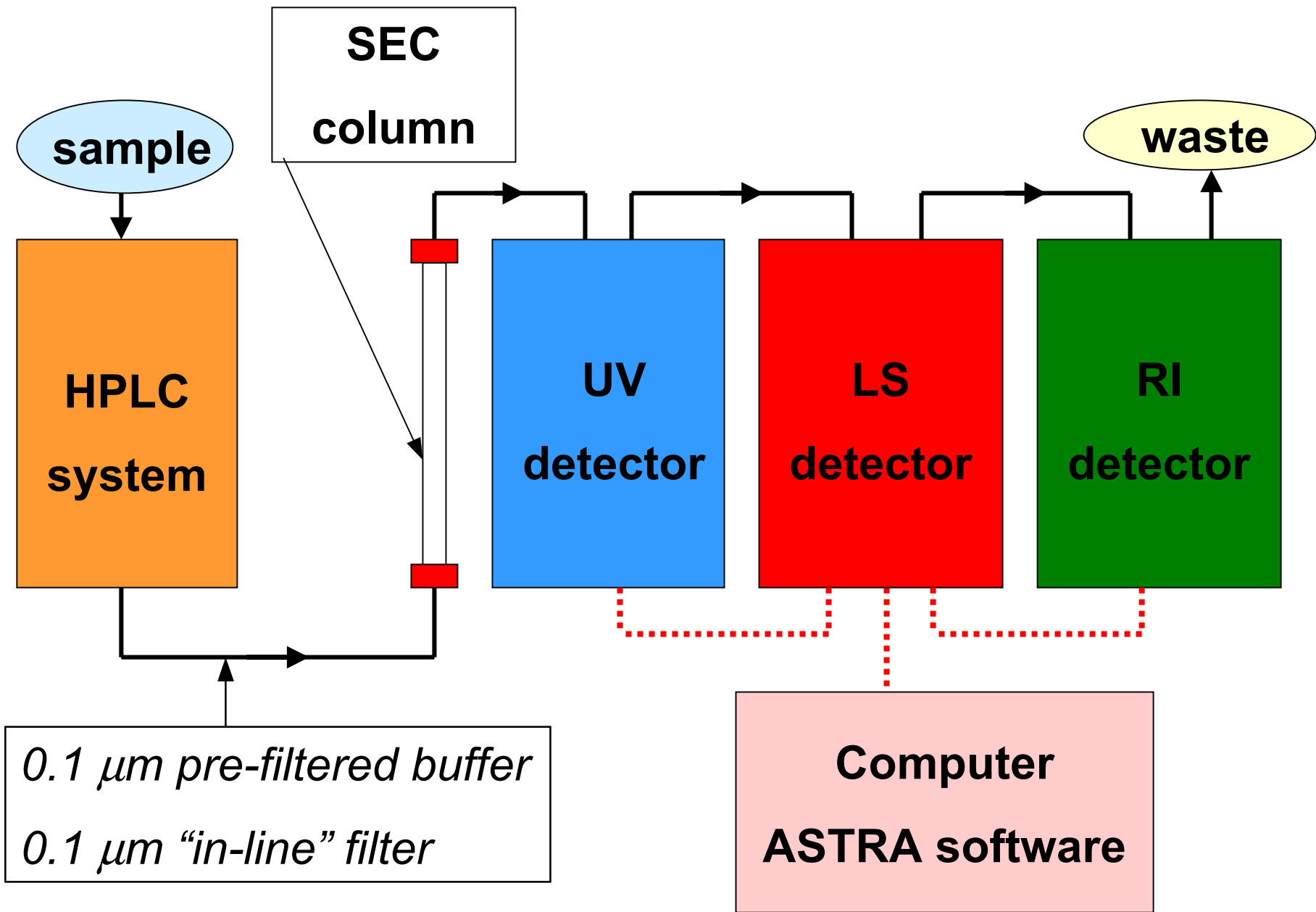
**Mw**  $(42.79 \pm 0.03) \times 10^3$  g/mol

Radius :  $0.0 \pm 0.0$  nm



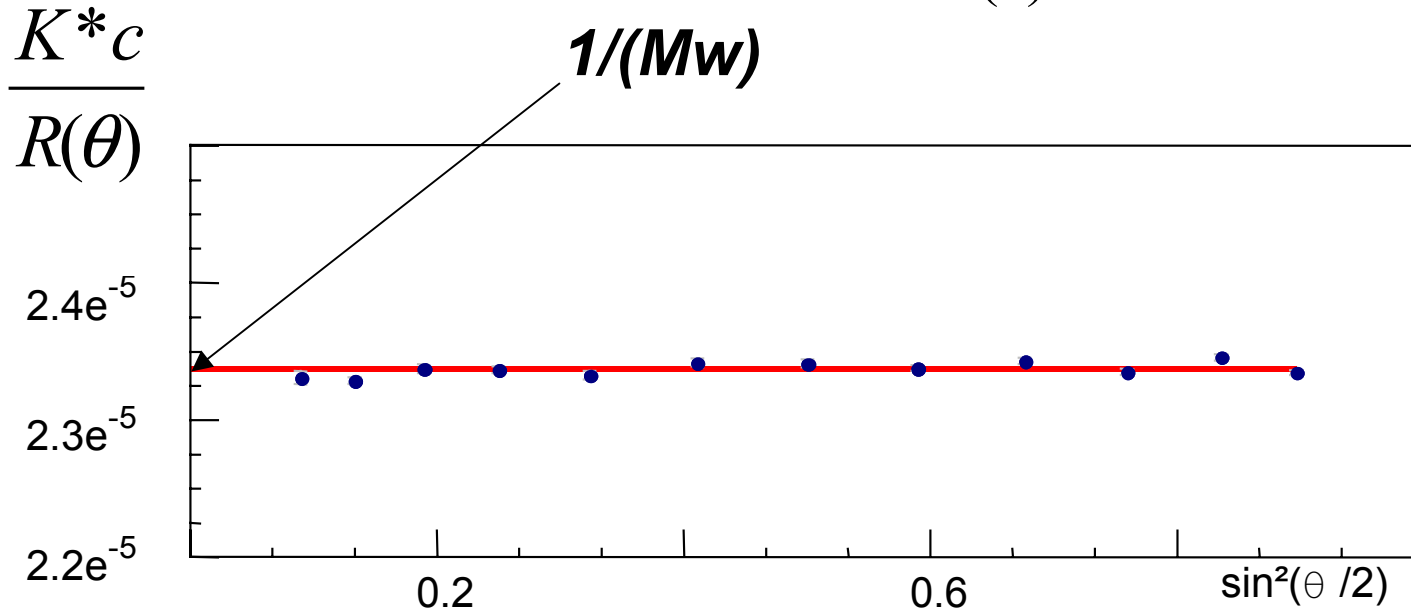
# ***Static Light Scattering***

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



# Zimm Plot Ovalbumin (43 kDa)

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

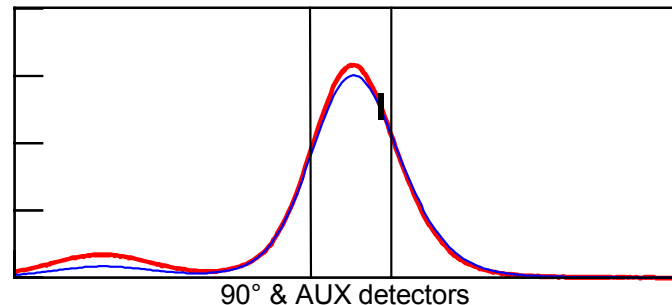


Volume : 16.300 mL

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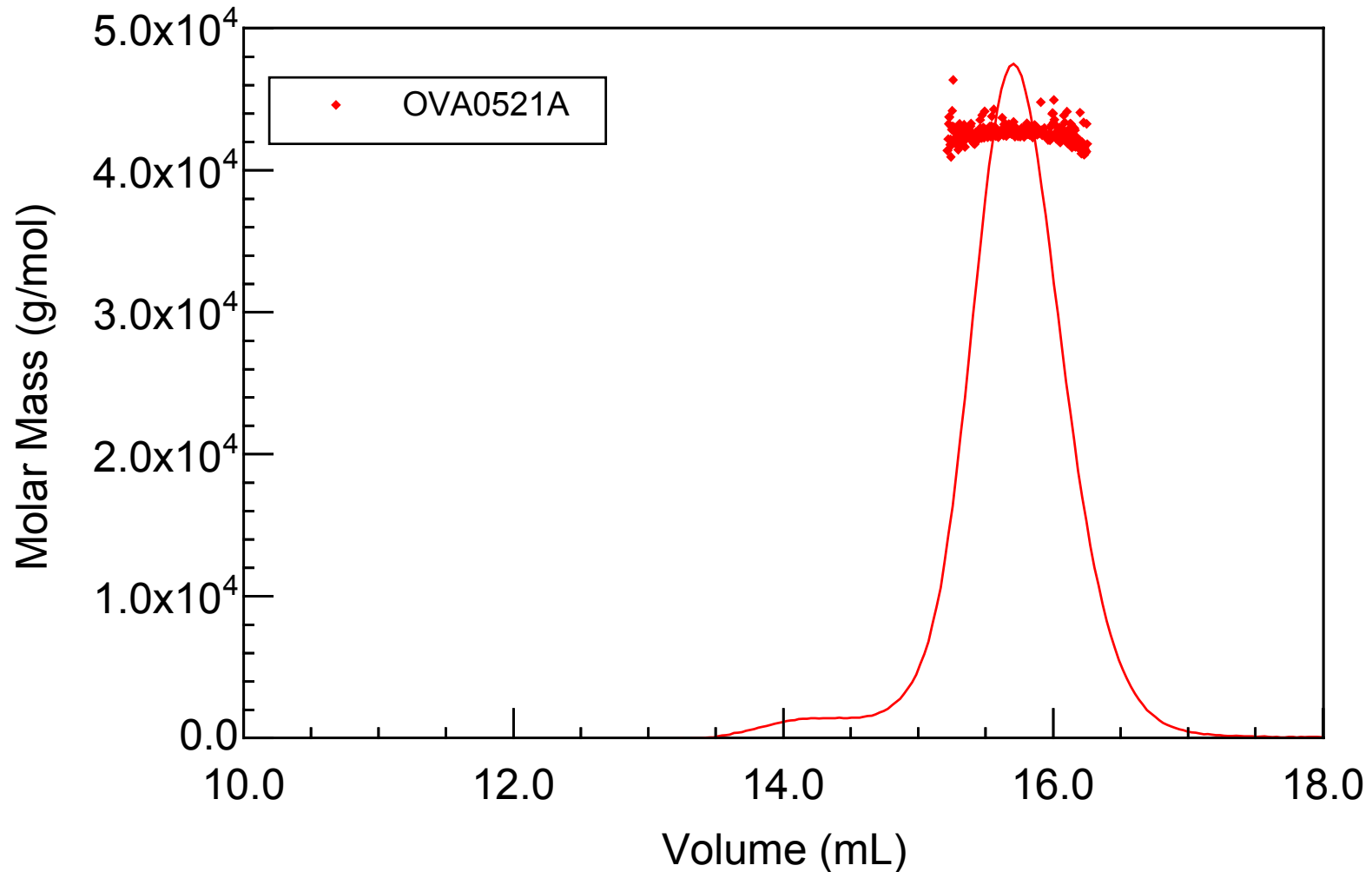
Radius :  $0.0 \pm 0.0$  nm



# ***Molar Mass Distribution Plot***

*Ovalbumin 43 kDa*

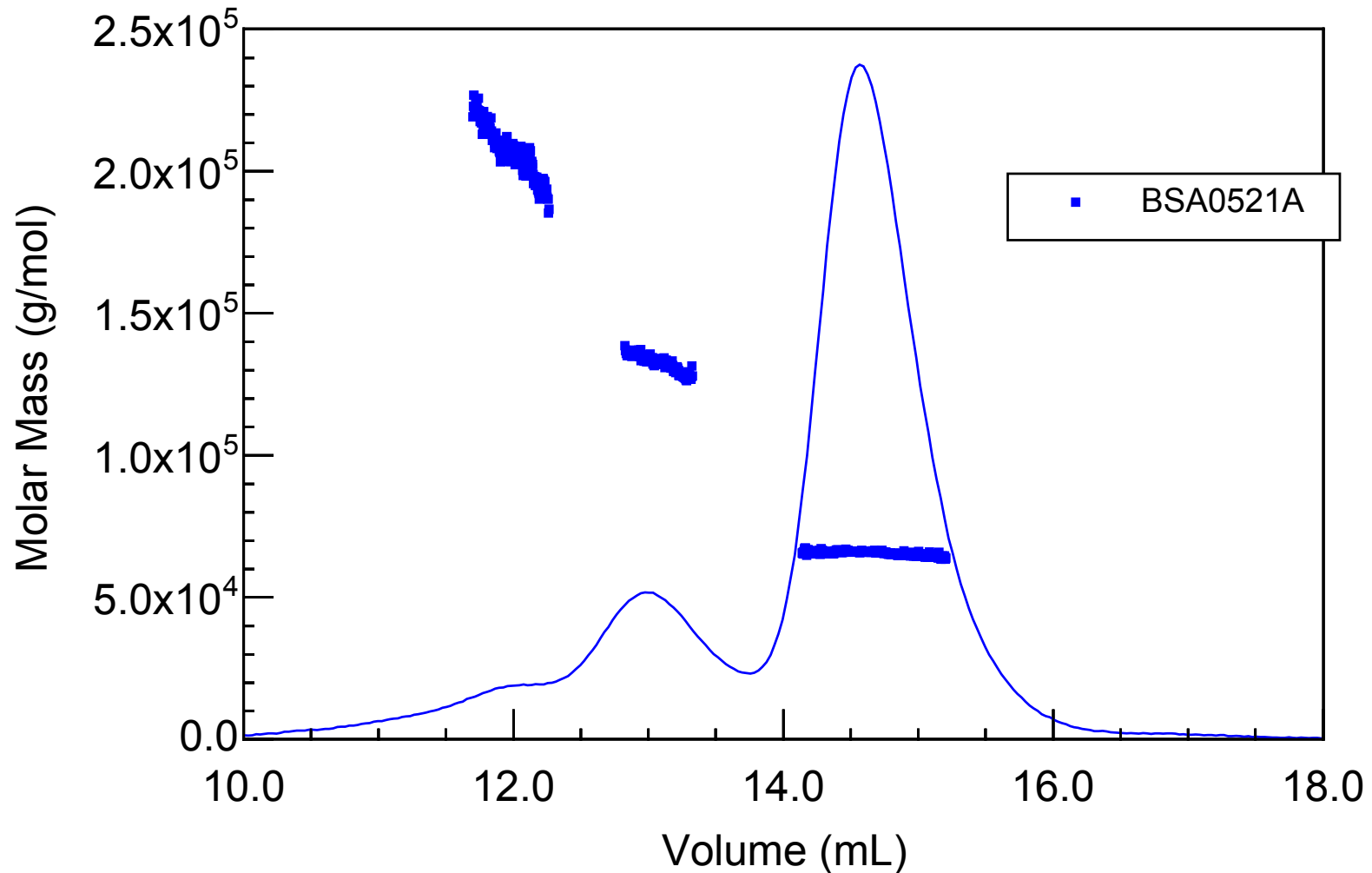
Molar Mass vs. Volume



# ***Molar Mass Distribution Plot***

*BSA 66 kDa*

Molar Mass vs. Volume



# ***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) <sup>a</sup>	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-Lactoglobulin	monomer	2	18.3	20.06 ± 0.33	9.7
Trypsin 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 <sup>x</sup> monomer	2	475.9	470.3 ± 2.62	1.2
<b>Median error:</b>					<b>2.3</b>

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***

<b>Protein</b>	<b>Amount loaded (<math>\mu\text{g}</math>)</b>	<b># Runs</b>	<b>Pred. MW (kDa)</b>	<b>Avrg. MW (kDa)</b>	<b>SD (kDa)</b>	<b>Avrg. error (%)</b>	<b>Range of accuracy (%)</b>
<b>Ovalbumin</b>	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
<b>Transferrin</b>	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<sub>SWXL</sub> [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.***

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

# ***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*

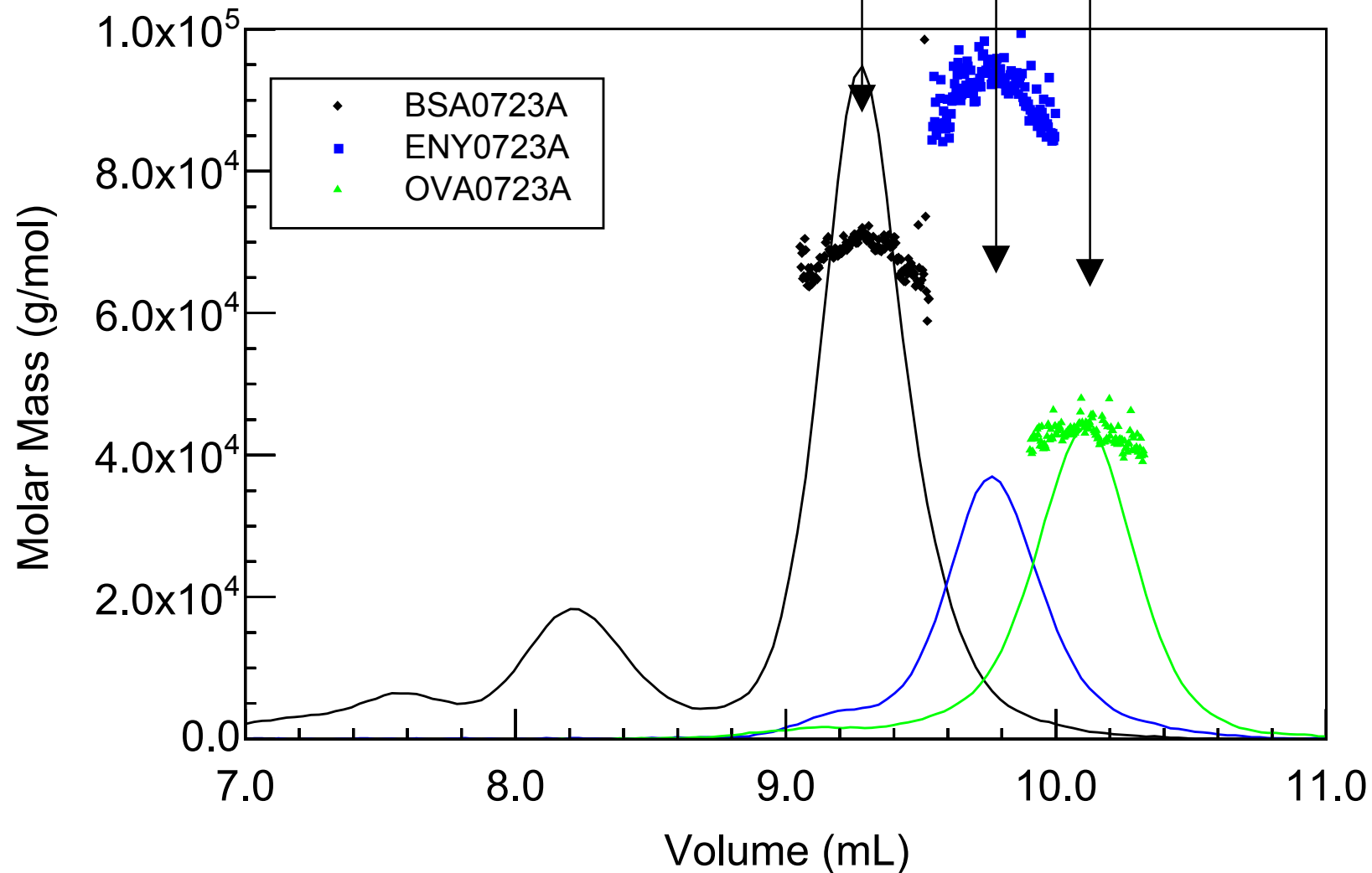
*Yeast Enolase - 93 kDa dimer  
(2x46kDa)*

***Enolase (Yeast) 46 kDa***

***OVA 43 kDa***

***dimer 93 kDa***

***BSA 66kDa***



# *Mixtures of non-interacting proteins*

## *Example:*

*BSA monomer - 66 kDa protein*

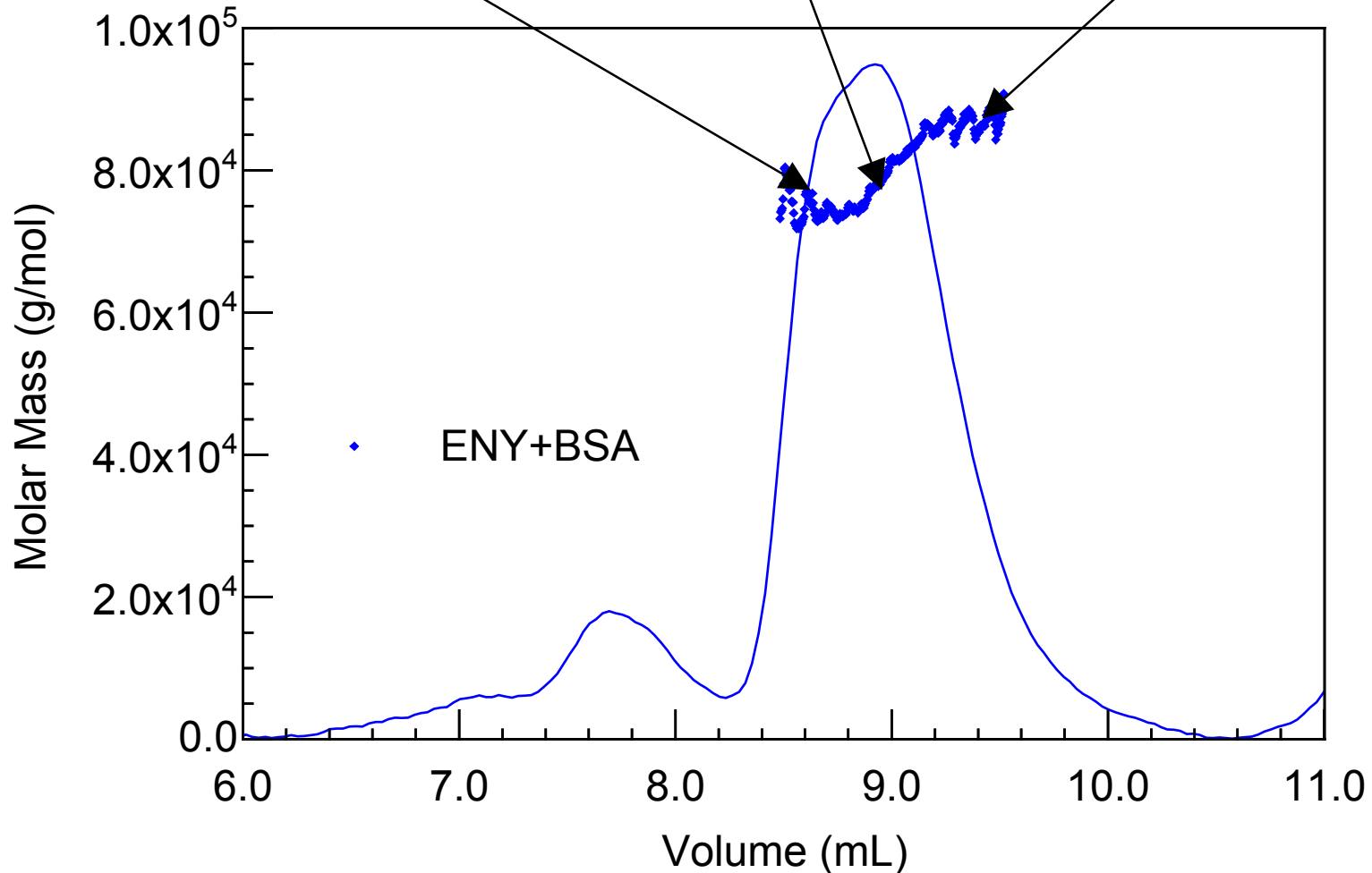
*Yeast Enolase - 93 kDa dimer  
(2x46kDa)*

# ***Analysis of co-eluting protein mixture***

***BSA 66kDa***

***BSA+ENY mixture***

***ENY dimer 93 kDa***



# *Analysis of interacting proteins*

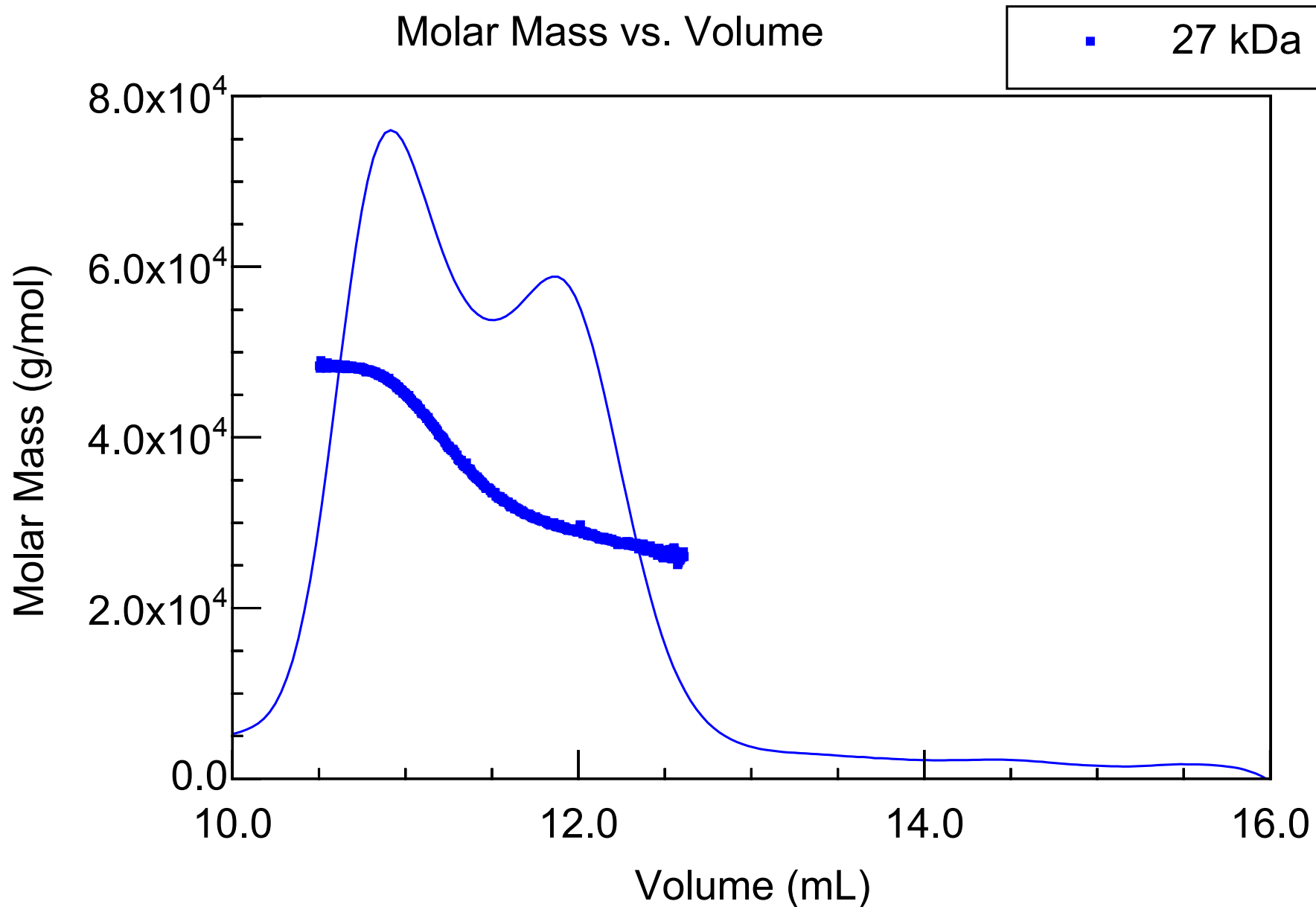
*Example:*

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

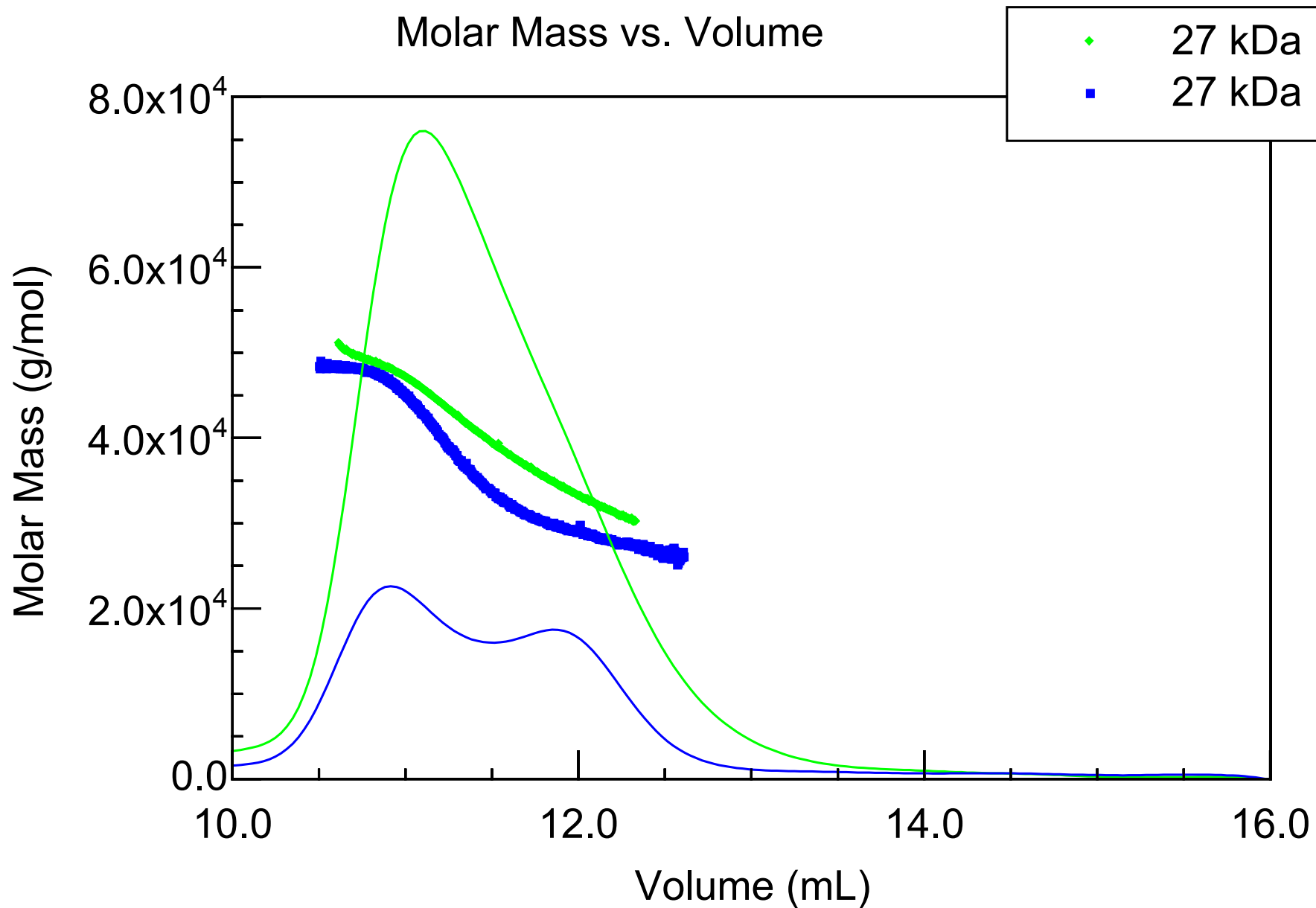
*ligand          7 kDa*

*Ligand binding shifts the protein into  
dimeric form*

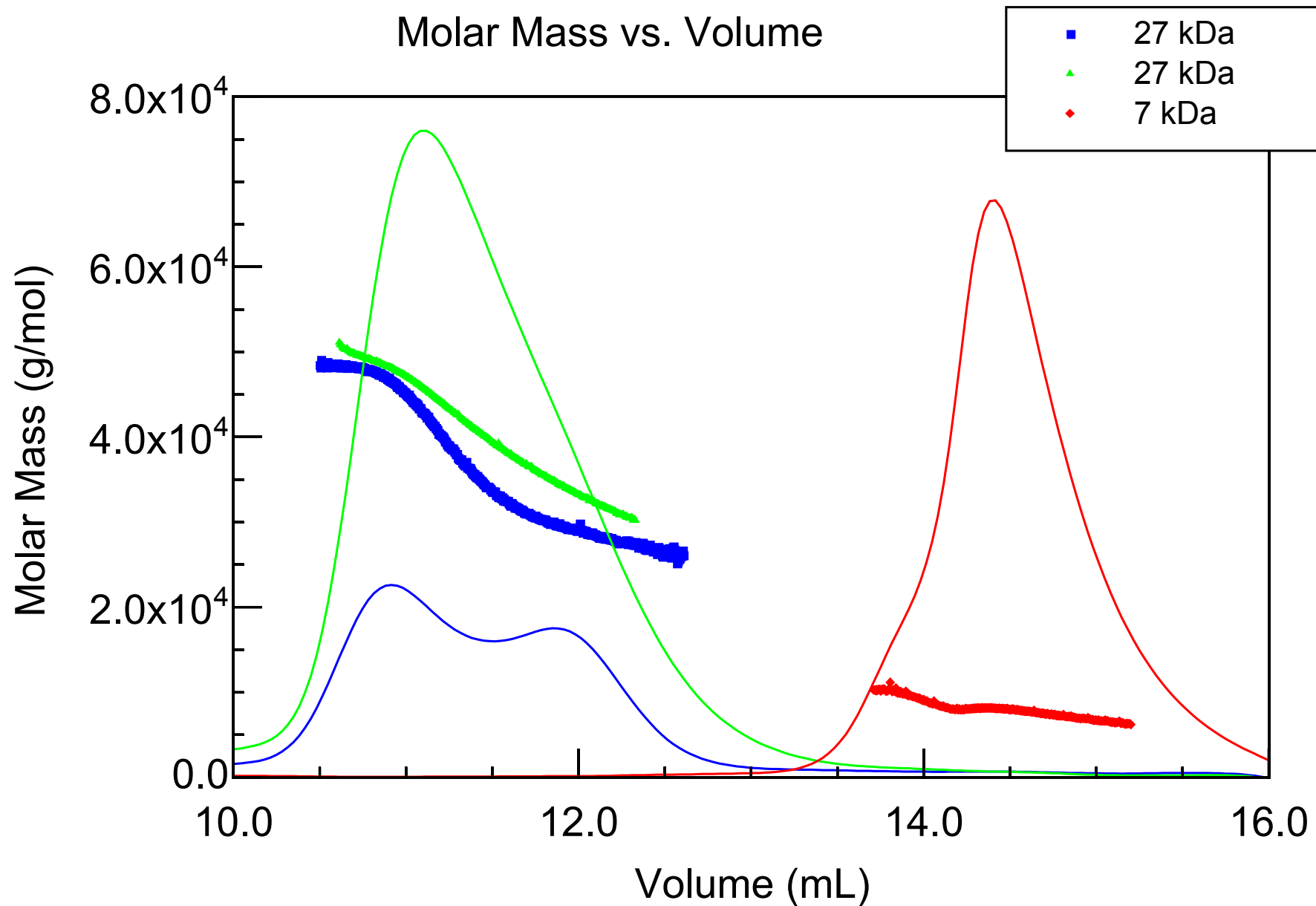
# ***Analysis of interacting proteins***



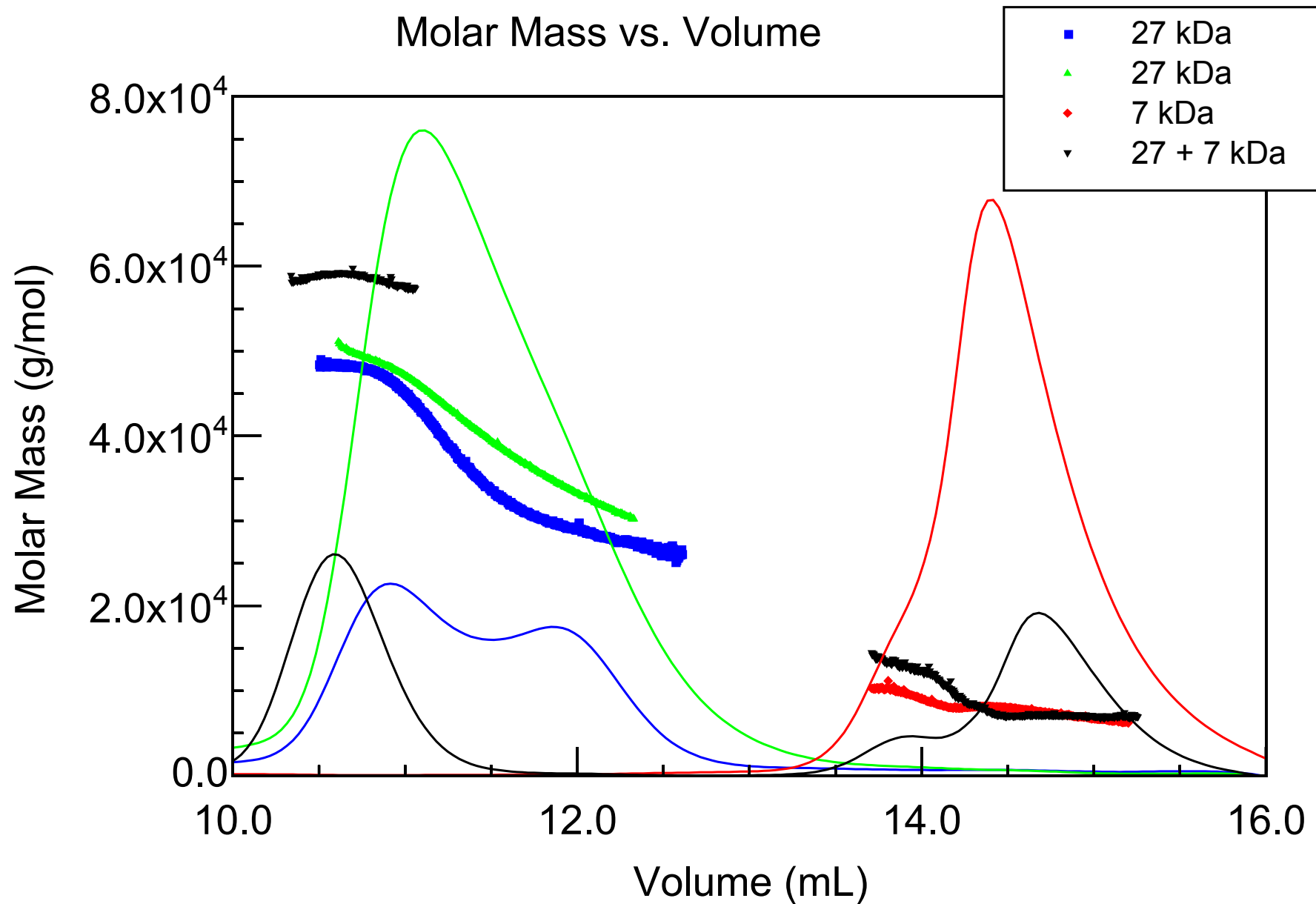
Molar Mass vs. Volume



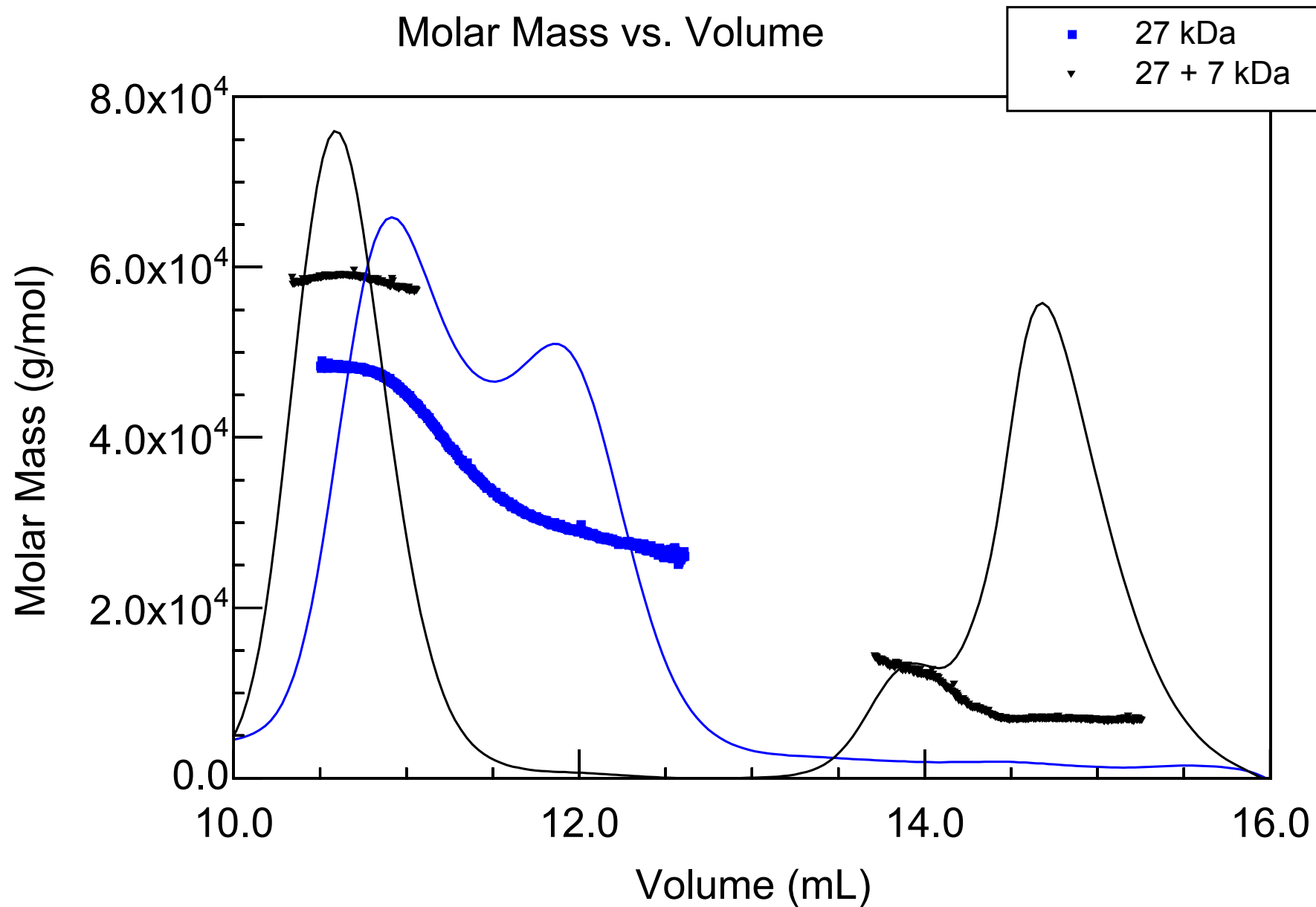
Molar Mass vs. Volume



Molar Mass vs. Volume



Molar Mass vs. Volume



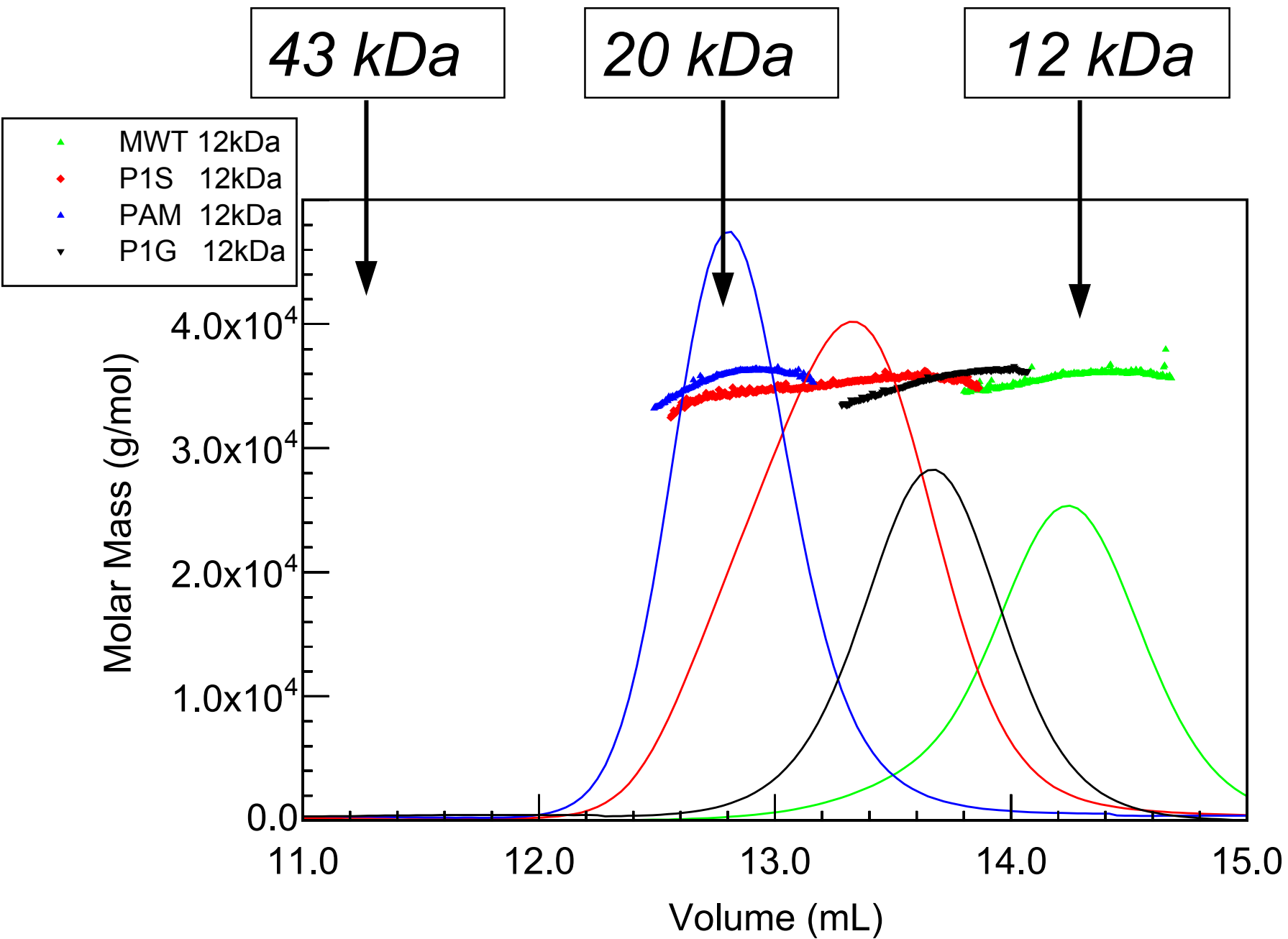
# ***Determination of the oligomeric state of mutant vs. wild type protein***

## ***Example:***

*protein 12 kDa (WT protein exists as a trimer)*

*Three mutants 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.*



# ***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  $(\langle r_g^2 \rangle^{1/2}) > (\lambda/20)$   
 $\sim 30 \text{ nm}$

## *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 ( $\mu\text{sec}$ ) with the initial ( $t=0$  sec) intensity

autocorrelator

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

***calculating the diffusion coefficient,  $D$***

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

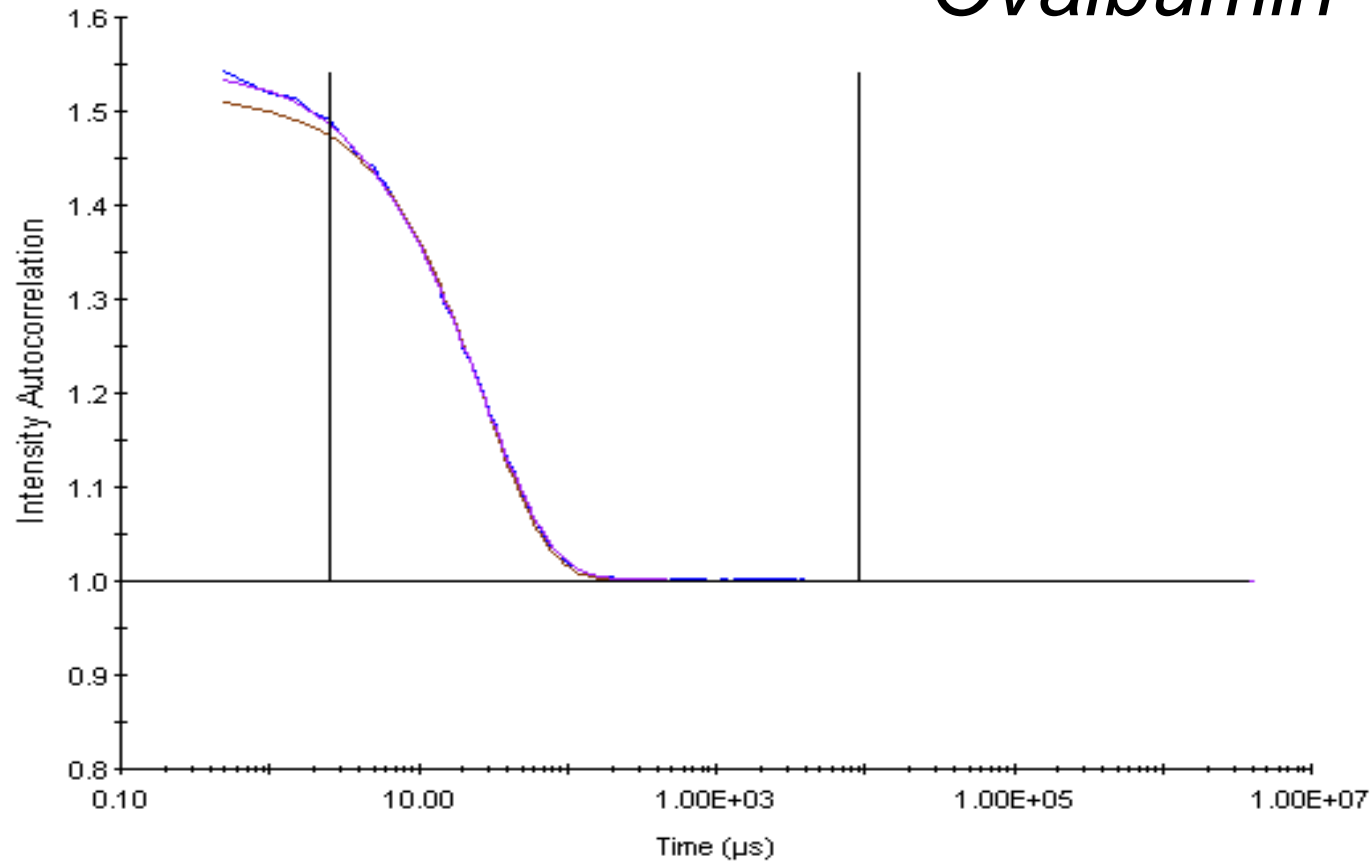
Stokes-Einstein equation

***MODEL: dilute system of spherical molecules***

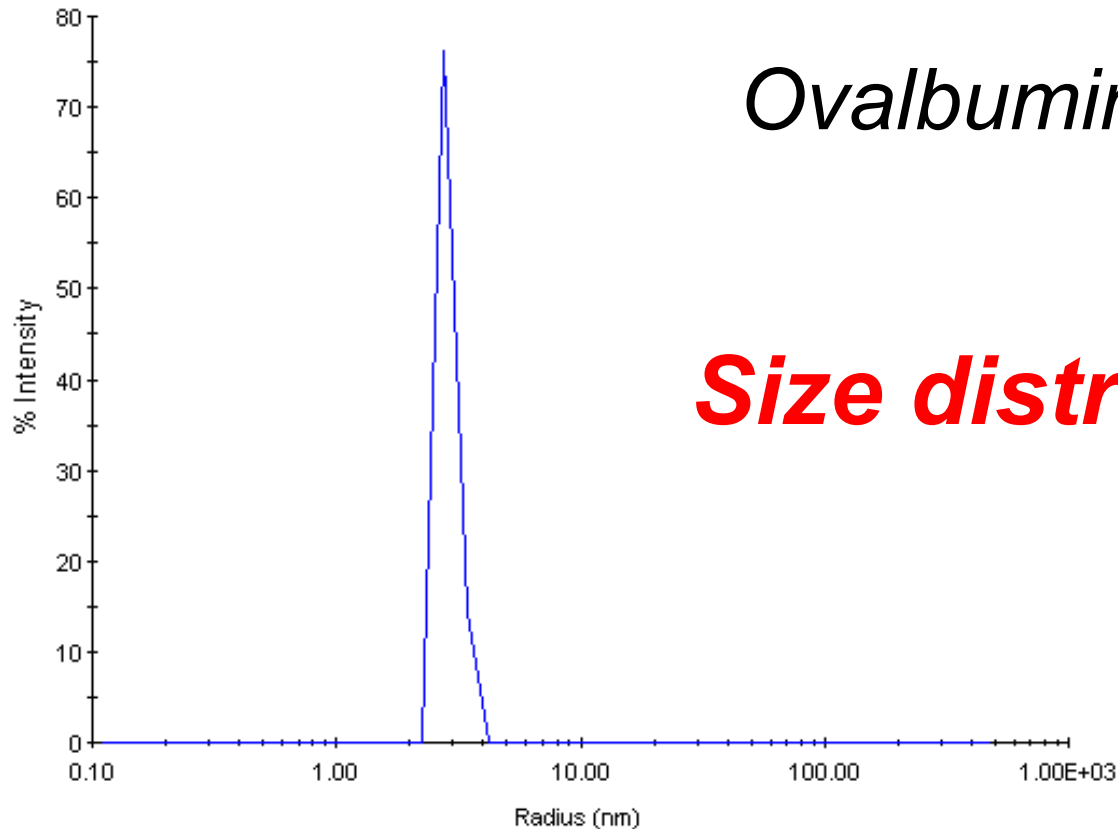
# Dynamic Light Scattering Experiments

## Autocorrelation function

Ovalbumin 43 kDa



# Dynamic Light Scattering Experiments



Ovalbumin 43 kDa

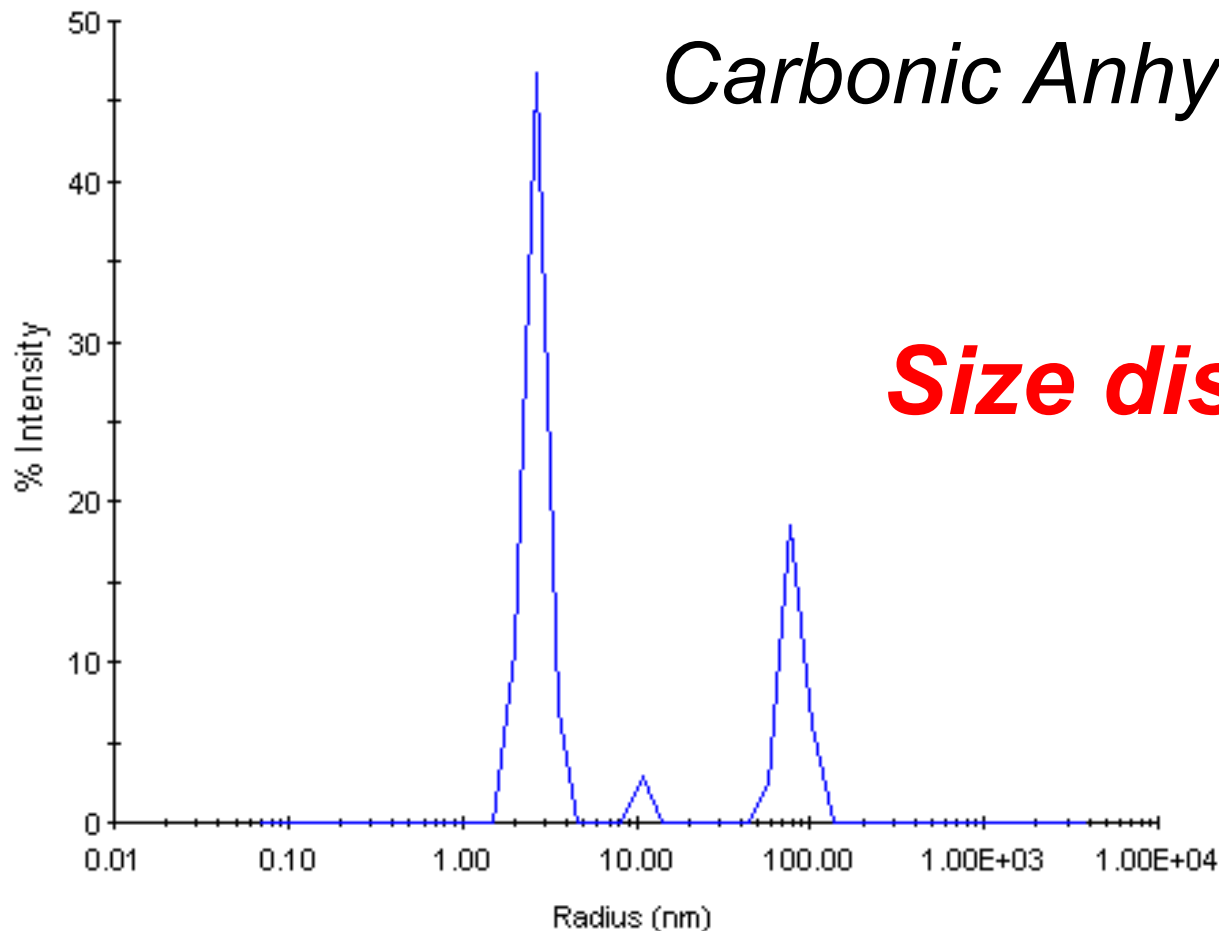
**Size distribution**

$R = 2.9 \pm 0.2 \text{ nm}$        $MW(R) = 40 \text{ kDa}$

*MW calculated from the calibration curve*

# Dynamic Light Scattering Experiments

*Carbonic Anhydrase 29 kDa*



***Size distribution***

$$R = 2.7 \pm 0.4 \text{ nm}$$

$$MW(R) = 33 \text{ kDa}$$

# ***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**

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

# ***Results obtained in “batch-mode” for polydisperse samples***

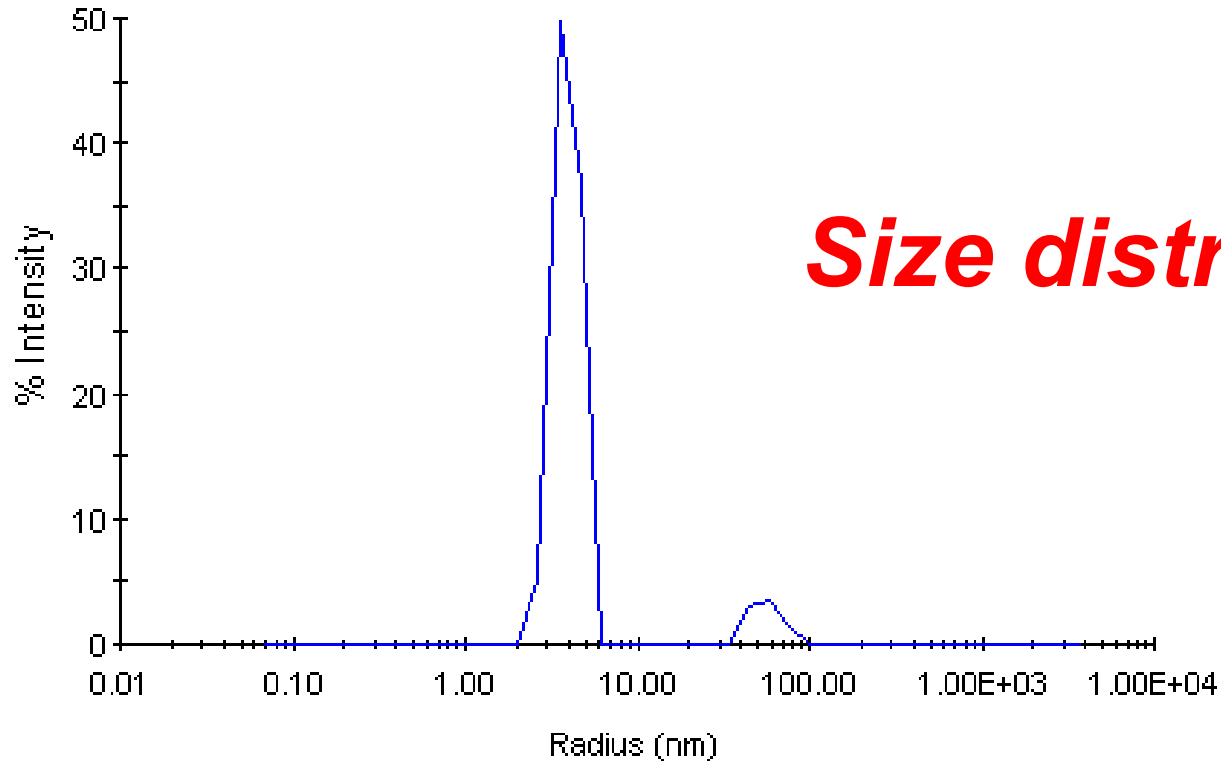
*In “batch-mode” the DLS experiment is able to detect that the sample is POLYDISPERSE (i.e. the sample is not homogeneous in respect to oligomeric state); it cannot however discriminate what oligomeric form are present*

*Example:*

*BSA : mixture of monomer, dimers*

# *Dynamic Light Scattering Experiments*

*BSA 66 kDa*

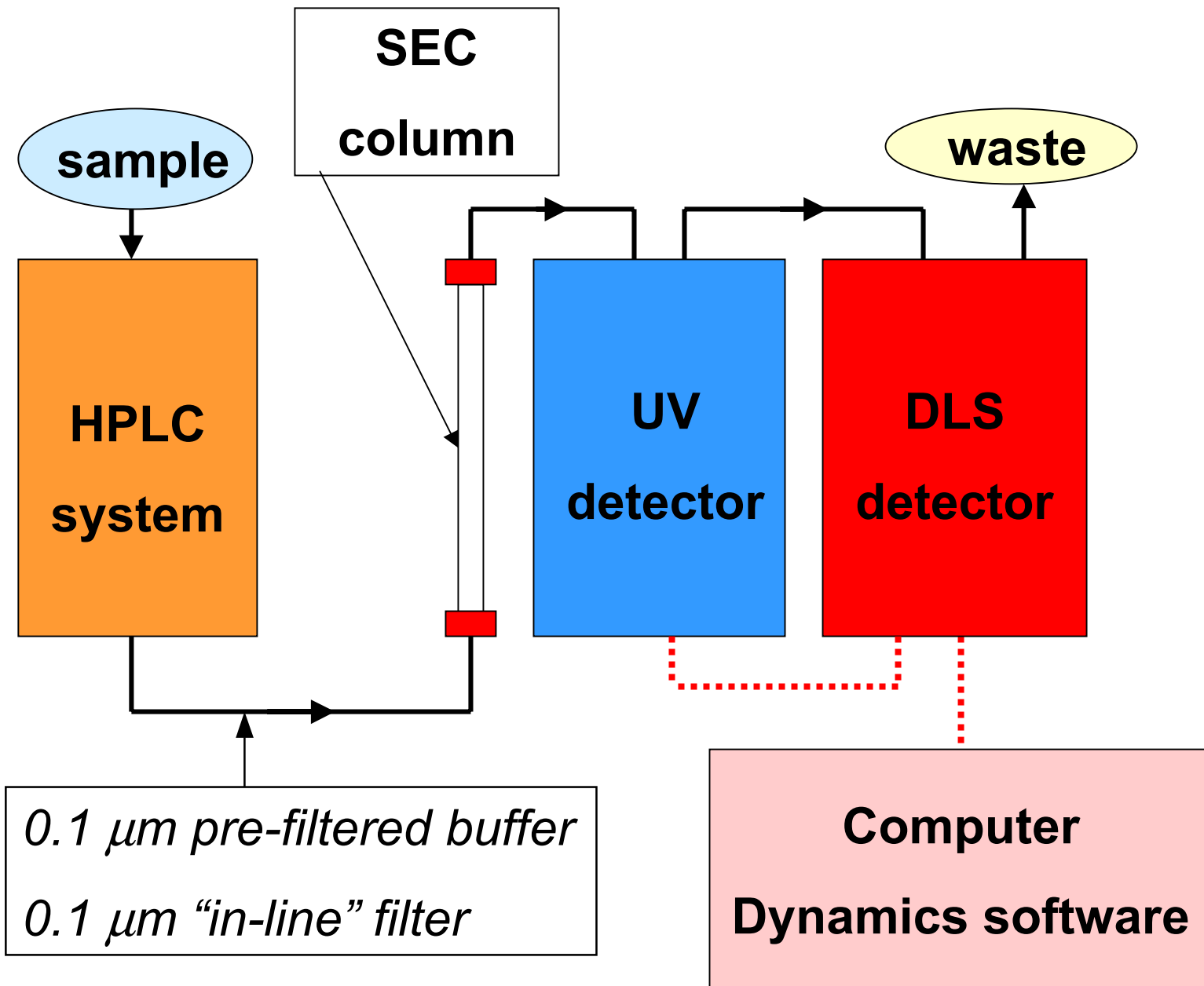


*$R = 4.0 \pm 0.6 \text{ nm}$*

*$MW(R) = 84 \text{ kDa}$*

# ***Dynamic Light Scattering***

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



## ***Results obtained in “SEC/LS” mode for polydisperse samples***

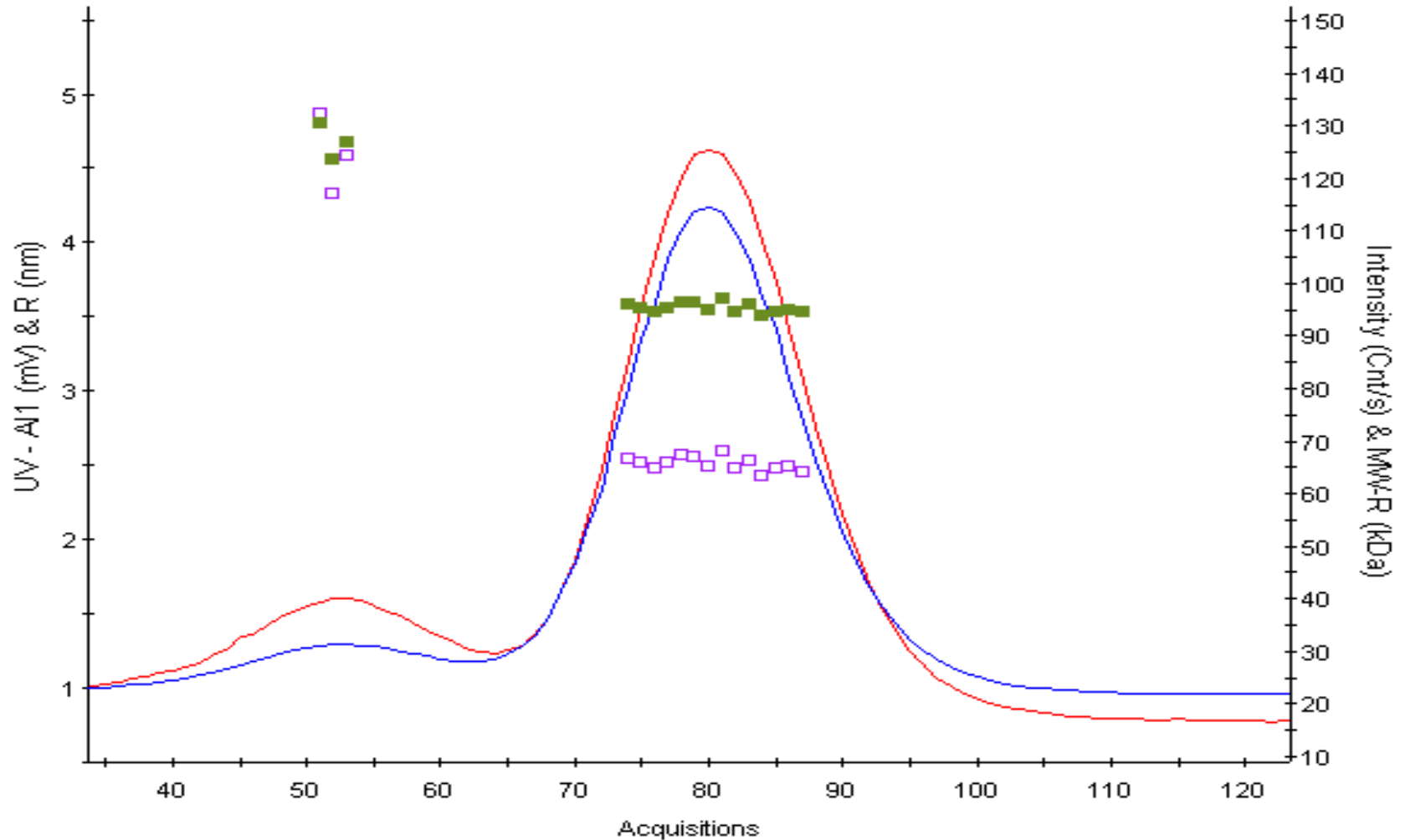
*In “SEC/LS” mode, the SEC serves as a fractionation step enabling determination of oligomeric state for each of the oligomeric forms that are present in the sample*

*Example:*

*BSA : mixture of monomer, dimers*

# Molar Mass Distribution Plot

BSA 66 kDa



— UV - AI1 (mV) X 1.00e-002  
— Intensity (Cnt/s) X 1.00e-003

■ R (nm)  
□ MW-R (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**

<b>Protein</b>	<b>Oligomeric state</b>	<b># Runs</b>	<b>Radius ± SD (nm)</b>	<b>Average MW (kDa)</b>	<b>Predicted MW (kDa)</b>	<b>Average error (%)</b>
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-Lactoglobulin	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
<b>Median:</b>						<b>20.0</b>

# *Hydrodynamic Radiuses and Molecular Weights Determined from “in-line” DLS analysis*

<b>Protein</b>	<b>Oligomeric state</b>	<b># Runs</b>	<b>Radius ± SD (nm)</b>	<b>Average MW (kDa)</b>	<b>Predicted MW (kDa)</b>	<b>Average error (%)</b>
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BSA (monomer)	monomer	3	3.56 ± .01	65.8	66.4	0.9
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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
<b>Median:</b>						<b>10.9</b>

# ***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  $\pm 5\%$
- 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  $\sim 10\text{-}20\%$  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*