# *Size Exclusion Chromatography Coupled with Light Scattering: Application to Study Proteins and Protein Complexes*

# *Size Exclusion Chromatography (SEC) Coupled with Light Scattering (LS)*

- *Derivation of Molecular Weight from LS experiment*
- *Experimental Set Up for SEC/LS "in-line"*
- *Evaluation of the SEC/LS System*
  - *Results for Standard Proteins*
  - *Sample Requirements*
- *Applications of SEC/LS to study protein complexes*
- *Conclusions*

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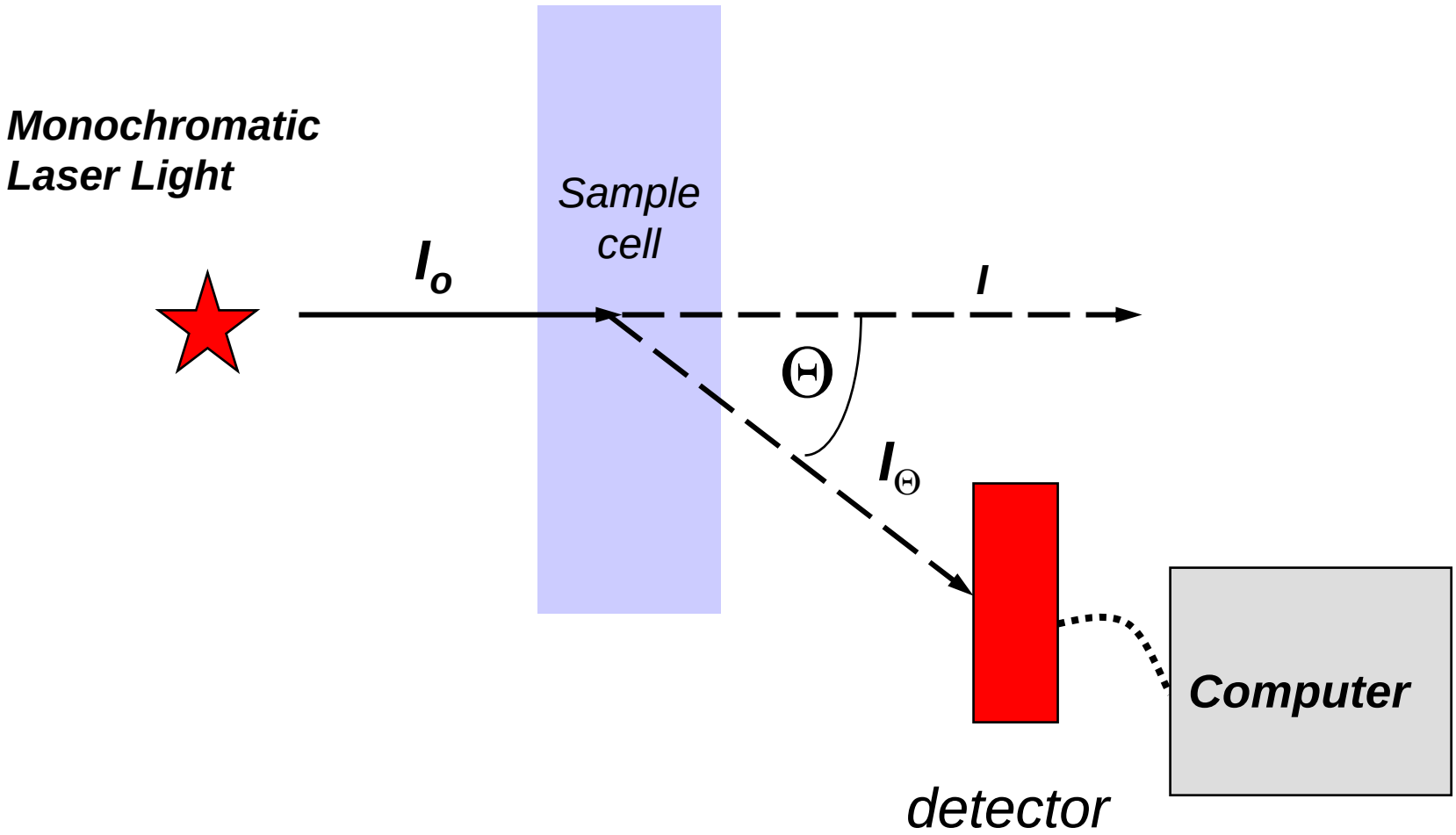
# *Light Scattering Experiments*



# Static Light Scattering Experiments

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

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

| | |
|---|---|
| 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 $4\pi^2 n^2 (dn/dc)^2 / (\lambda_0^4 N_A)$ |
| 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

at low concentrations c < 0.1 mg/mL

$$2A_2cMw<<1$$

thus, the second virial coefficient term ($2A_2c$) can be neglected

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

expansion of P(Θ) to the first order gives

$$1/P(\Theta) = 1 + (16\pi^2/3\lambda^2) \langle r_g^2 \rangle \sin^2(\Theta/2) + ....$$

# Static Light Scattering Experiments

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

Using a multi angle instrument

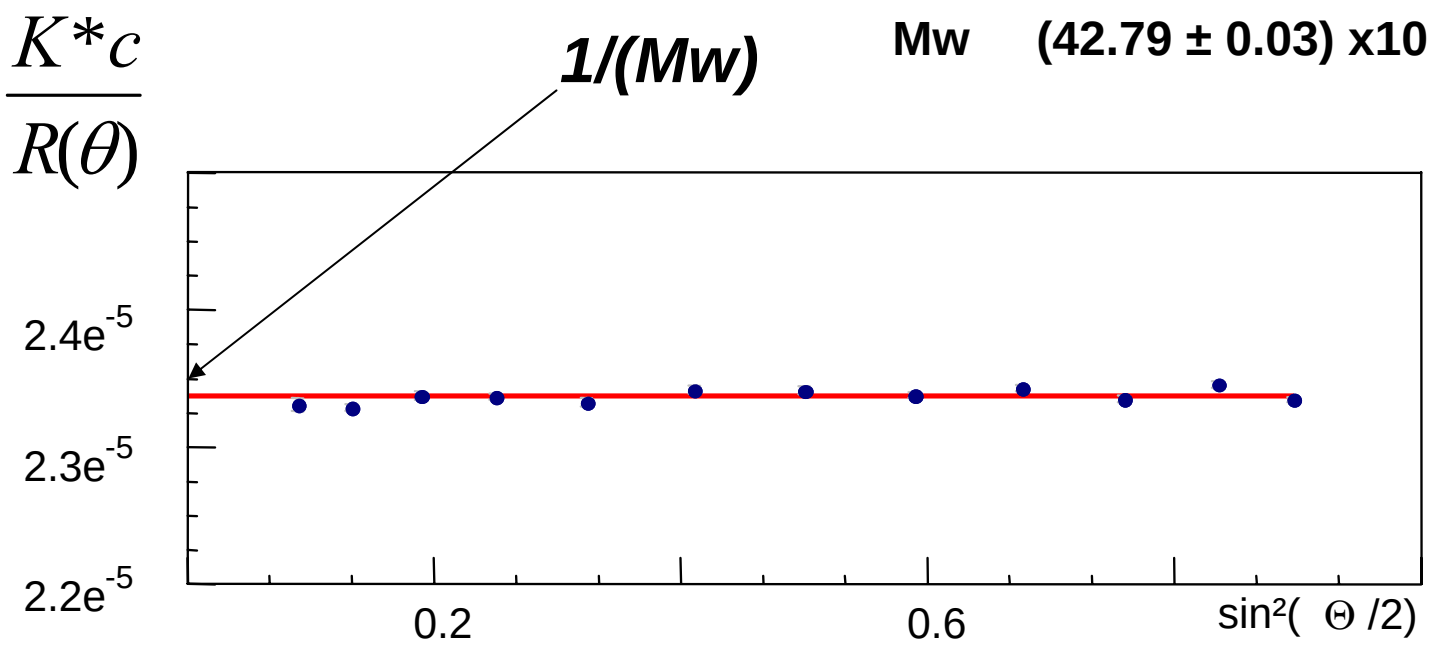
construct a plot of

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

***From intercept ⟶ Derived MW***

# Zimm Plot Ovalbumin (43 kDa)

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




**Mw (42.79 ± 0.03) x10 $^3$ g/ mol**

*At low concentrations*

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

***From intercept*** ⟶ ***Derived MW***

***weight-average MW***

***fractionate samples***

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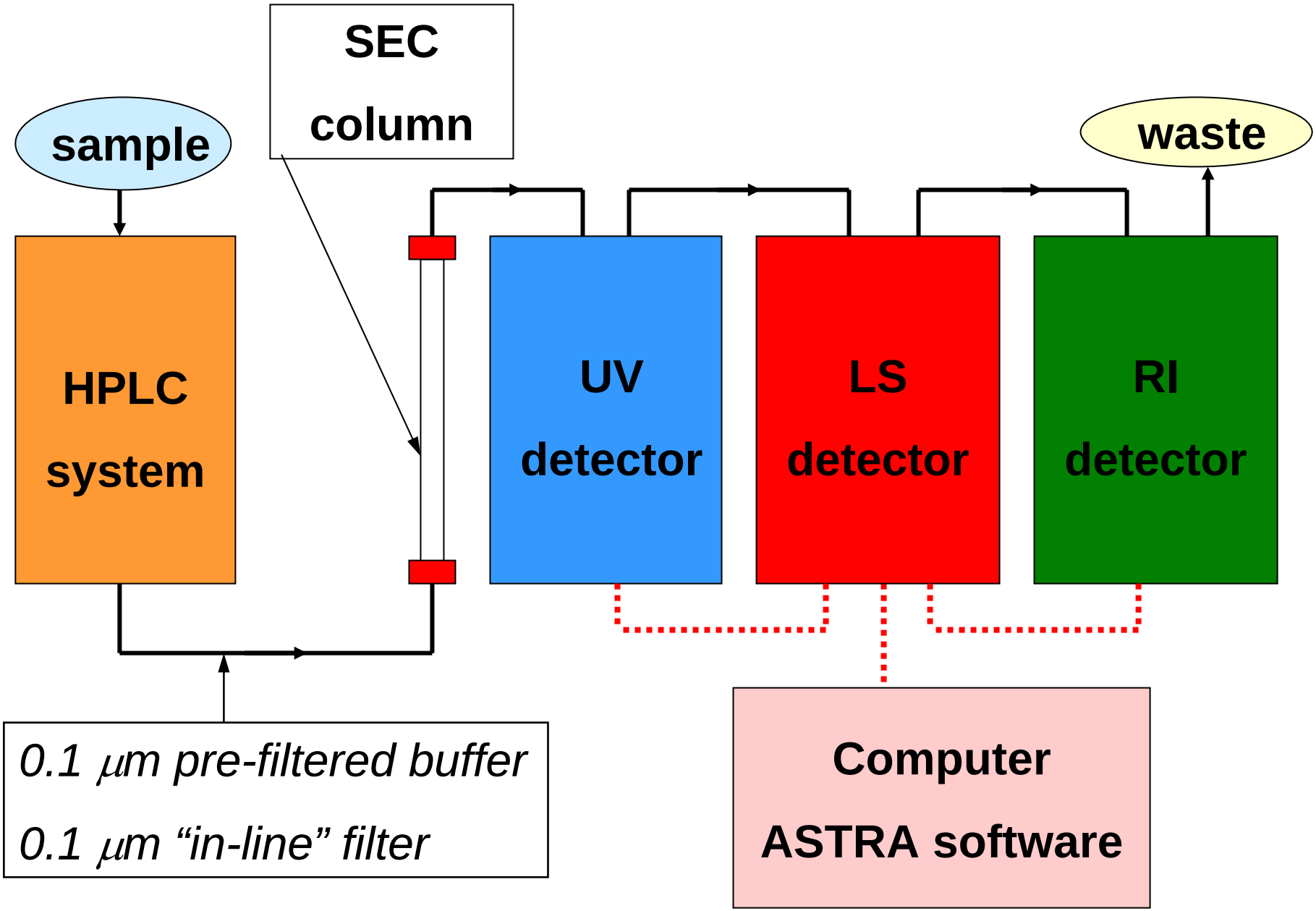
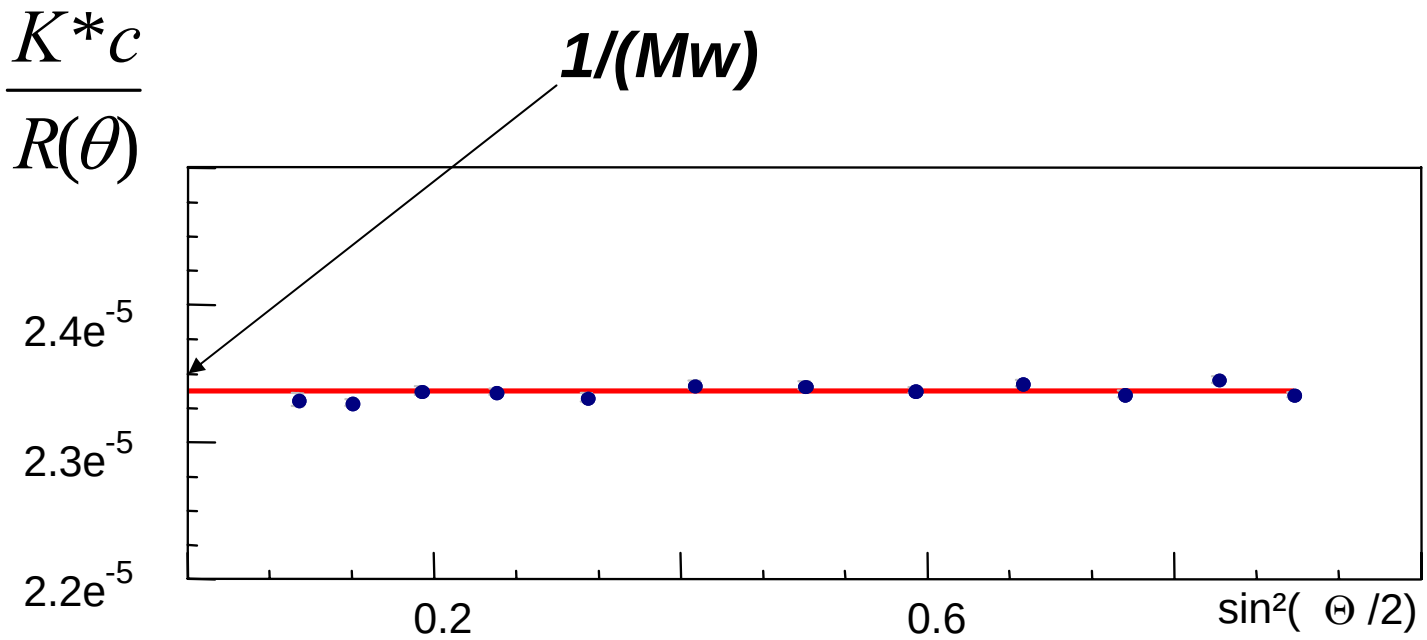

SEC
column
sample
waste
HPLC
system
UV
detector
LS
detector
RI
detector
0.1 μm pre-filtered buffer
0.1 μm "in-line" filter
Computer
ASTRA software

# Zimm Plot Ovalbumin (43 kDa)

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

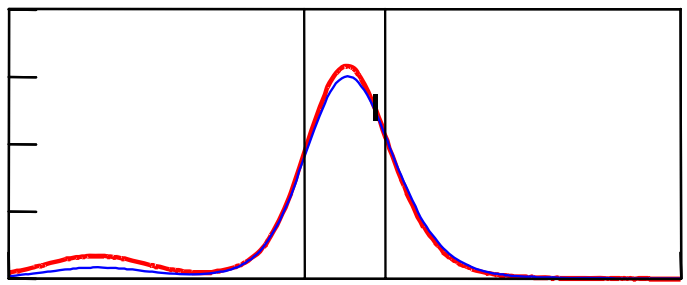



Volume : 16.300 mL

Conc. : (0.173 ± 0.000) mg/mL

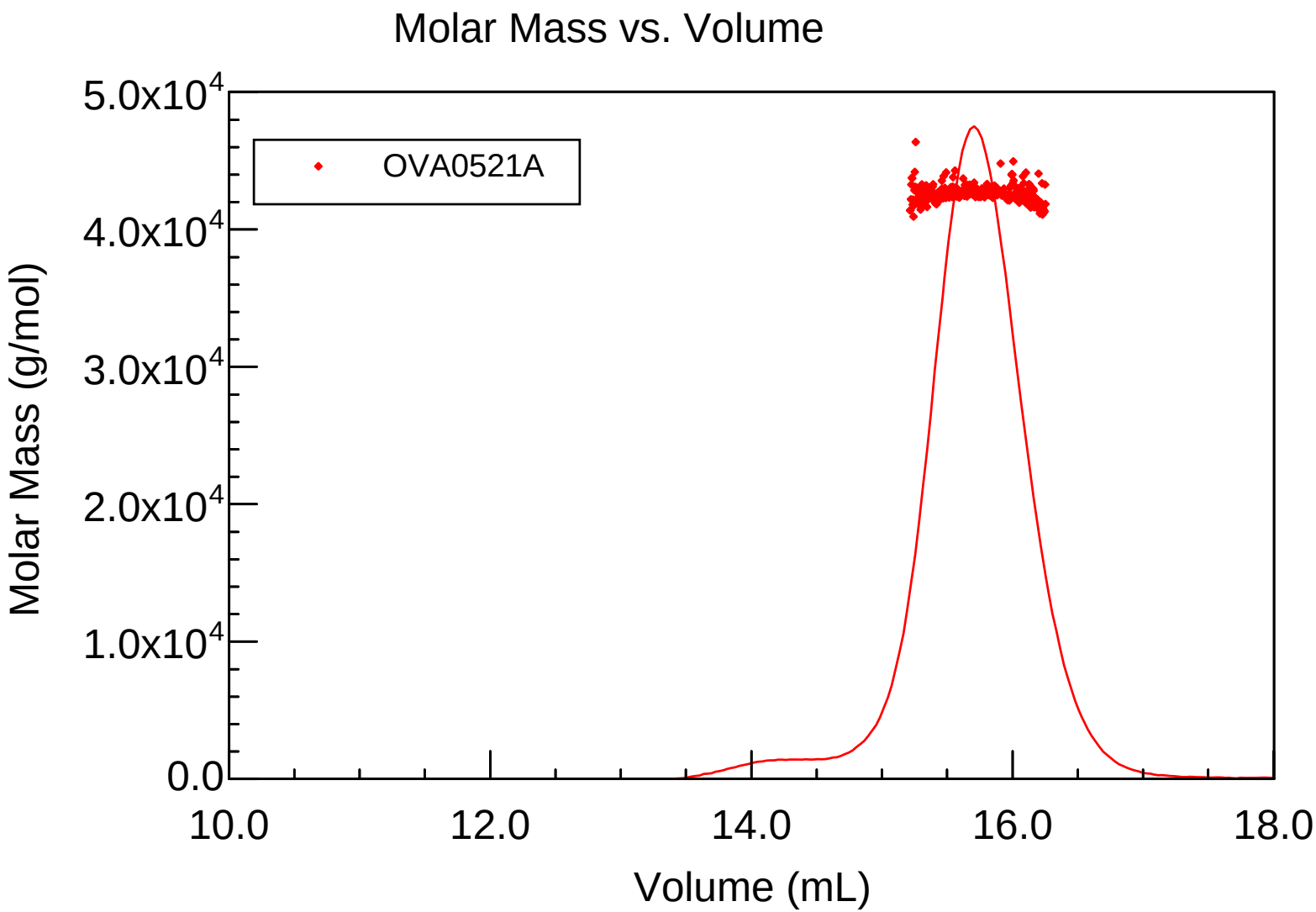
**Mw (42.79 ± 0.03) x10$^3$ g/mol**

Radius : 0.0 ± 0.0nm



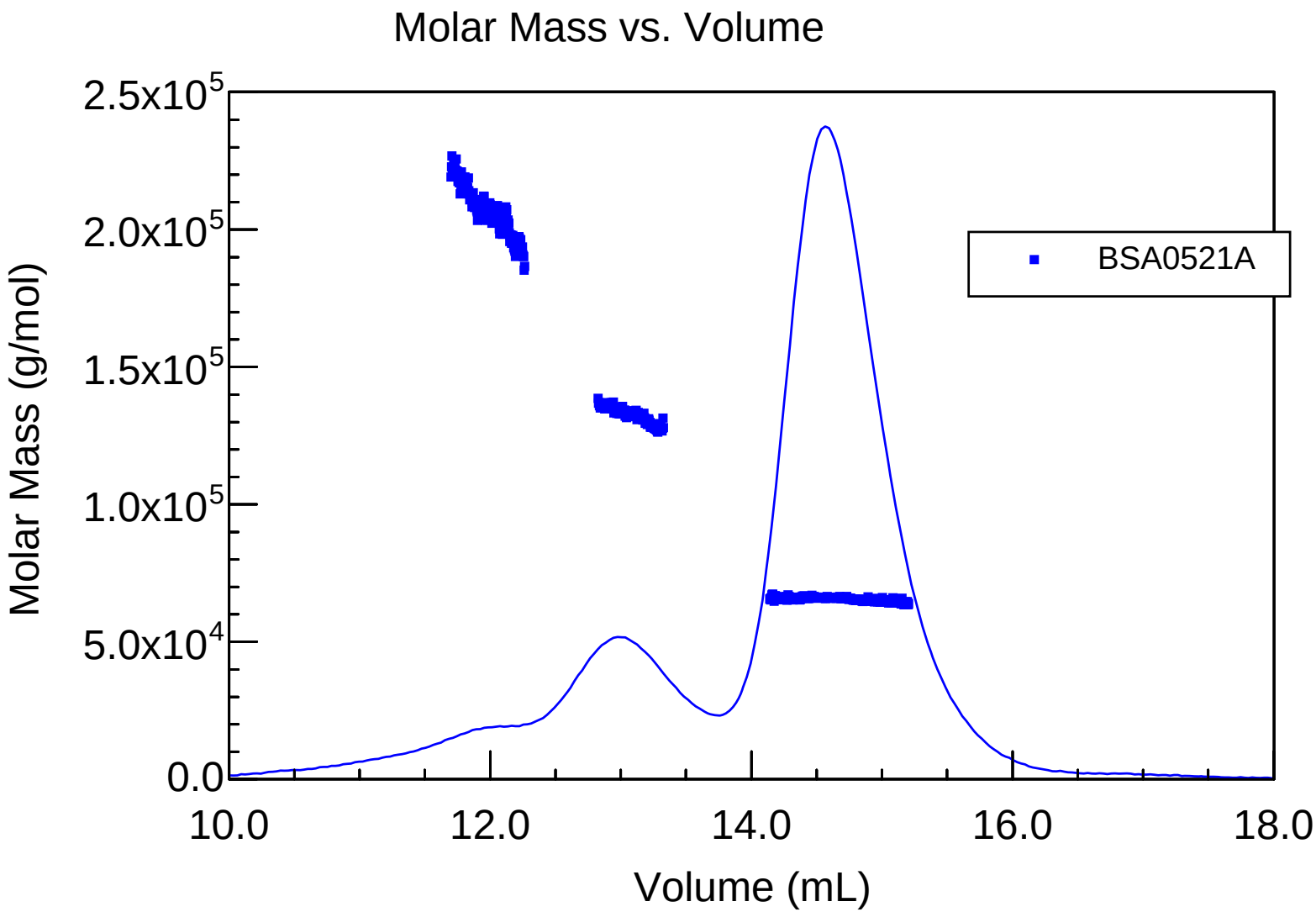
# Molar Mass Distribution Plot

*Ovalbumin 43 kDa*



# Molar Mass Distribution Plot

*BSA 66 kDa*



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## *Molecular Weights Determined from "in line" analyses; static LS with SEC in line; 16 protein standards, MW 6.5 to 475 kDa*

| 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 |
| βLactglobulin | 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

# *Sample Requirements for Proteins*

| **Column** | **Optimal amount of protein µg [$10^{-6}$ g]** | | | |
|---|---|---|---|---|
| | **MM >200 kDa** | **MM 40-200 kDa** | **MM 10-40 kDa** | **MM <10 kDa** |
| **Superose 6 HR 10/30** | **50** | **50-100** | Not suitable | Not suitable |
| **Superdex 200 HR 10/30** | 50 | **50-100** | **100-200** | Not suitable |
| **Superdex 75 HR 10/30** | Not suitable | 50-100 | **100-200** | Not suitable |
| **Superdex peptide HR 10/30** | Not suitable | Not suitable | Not suitable | **400-800** |

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# *Applications of SEC/LS to study protein complexes*

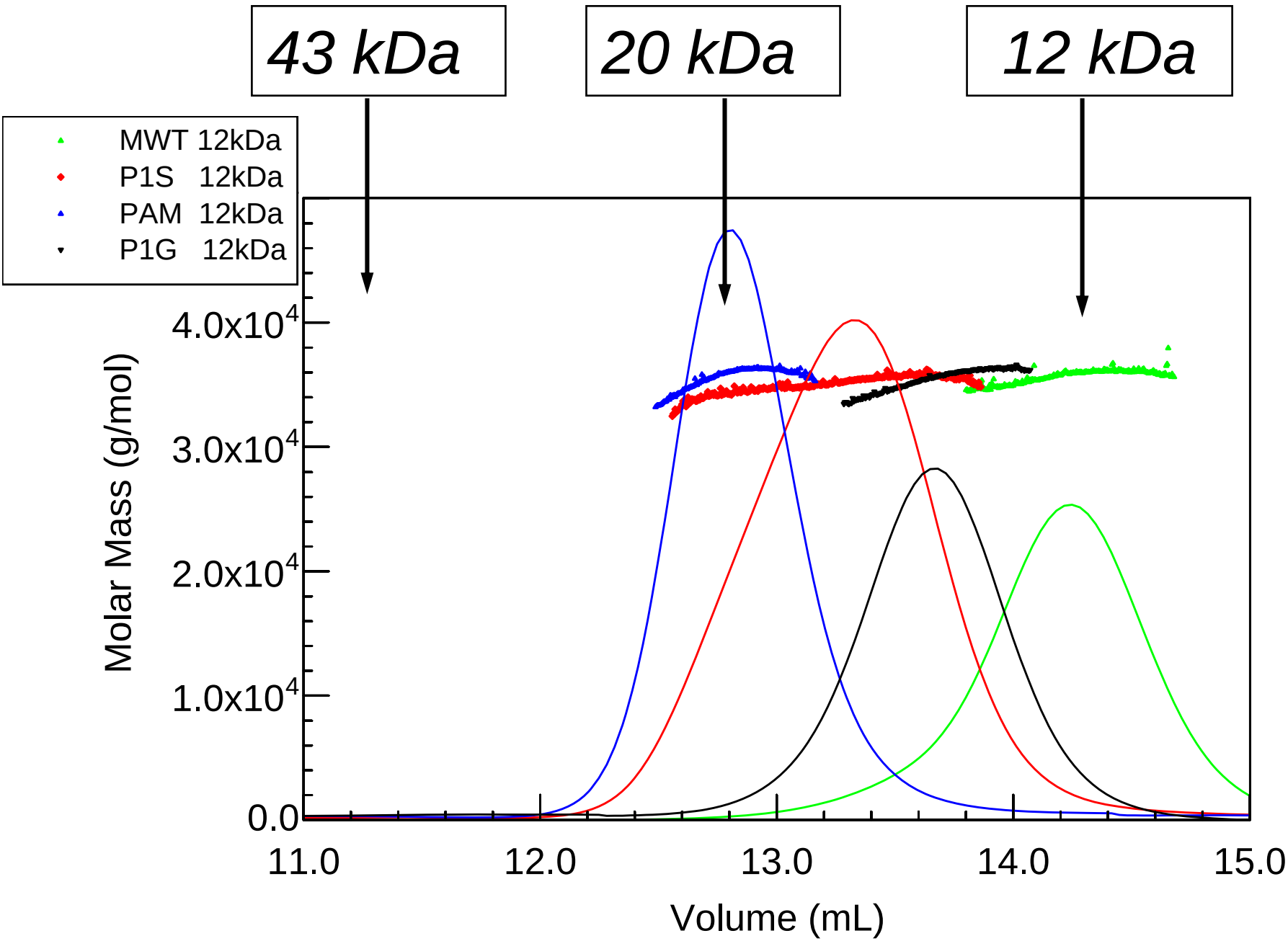
- *Determination of the oligomeric state of mutant vs. wild type protein*
- *Mixtures of non-interacting proteins*
- *Mixtures of interacting protein- detection of ligand driven protein complexes*
- *Determination of oligomeric state of membrane proteins solubilized in detergents*

# *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.*


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)

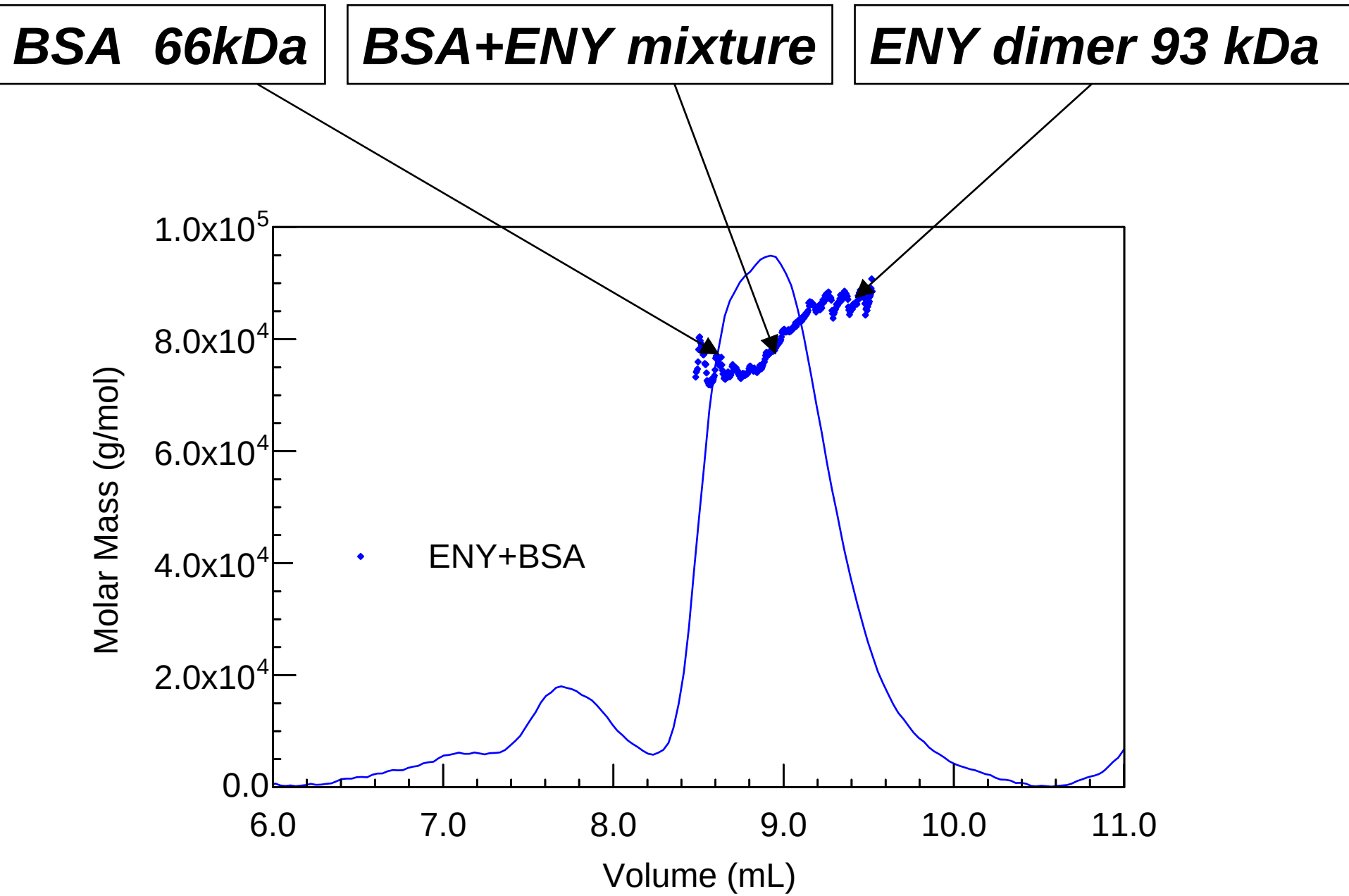
# *Mixtures of non-interacting proteins*

*Example:*

*BSA monomer - 66 kDa protein*

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

# Analysis of co-eluting protein mixture



# *Mixtures of interacting protein-detection of ligand driven protein oligomerization*

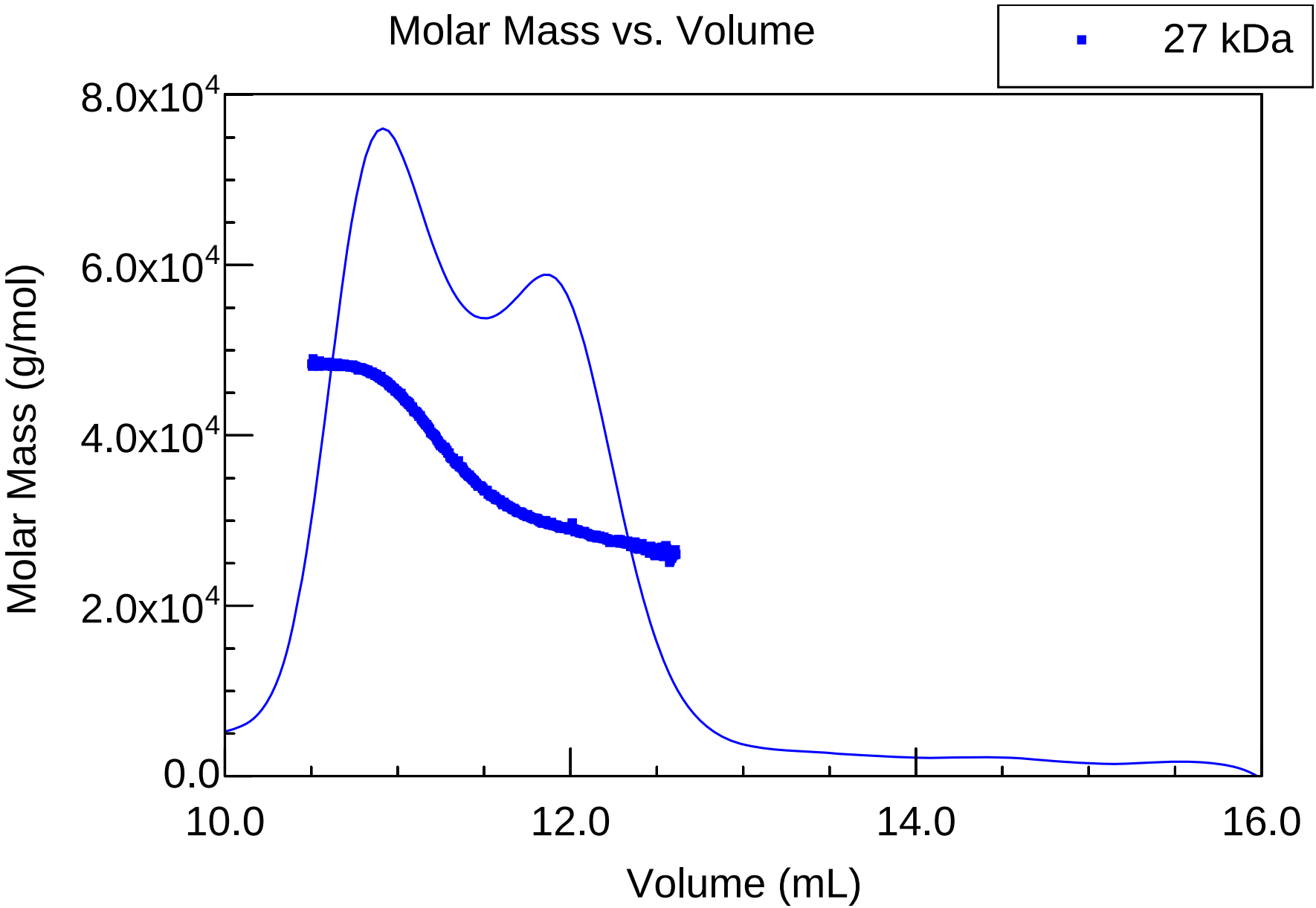
*Example:*

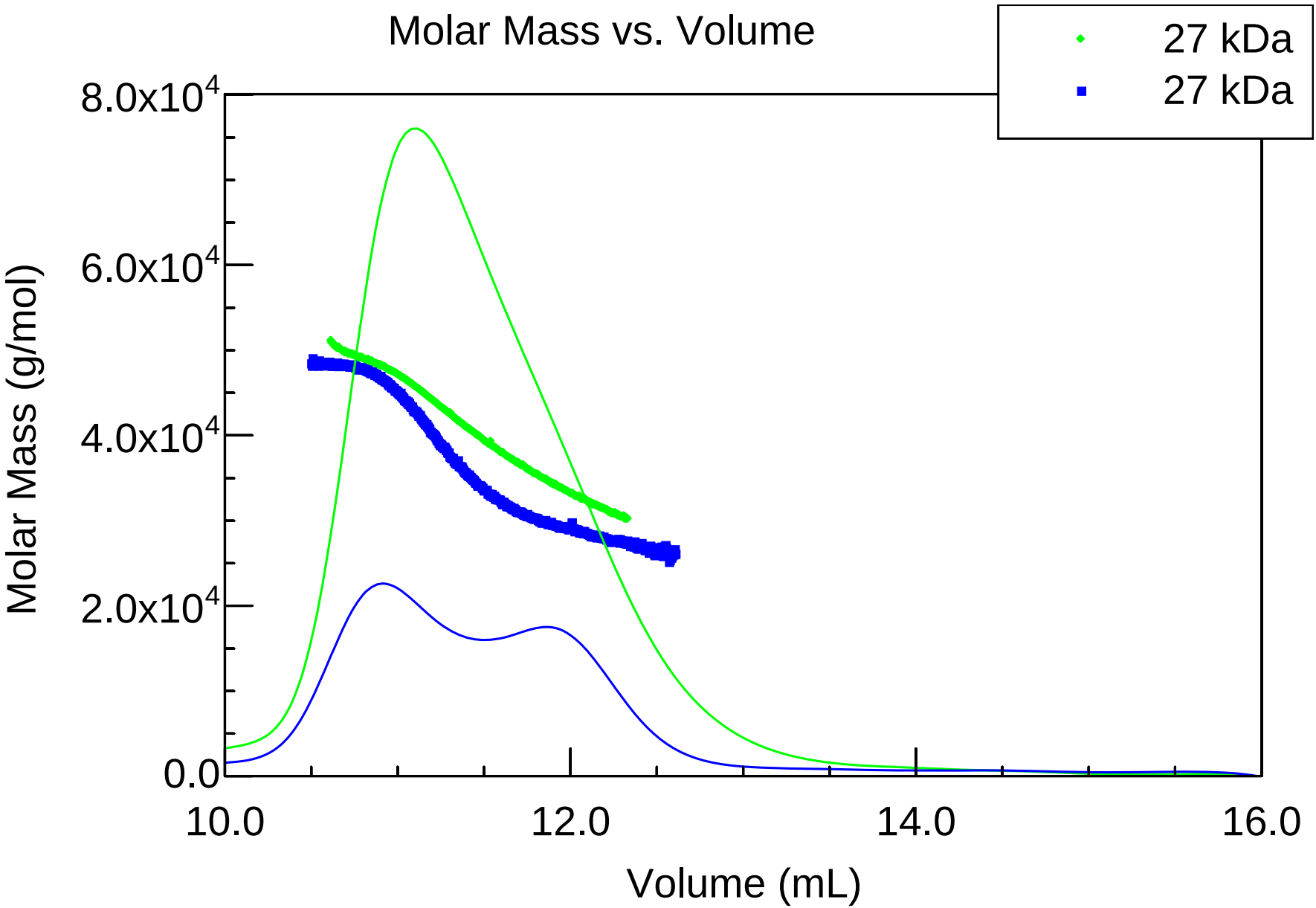
*protein 27 kDa (protein exists as a mixture of monomer and dimer)*

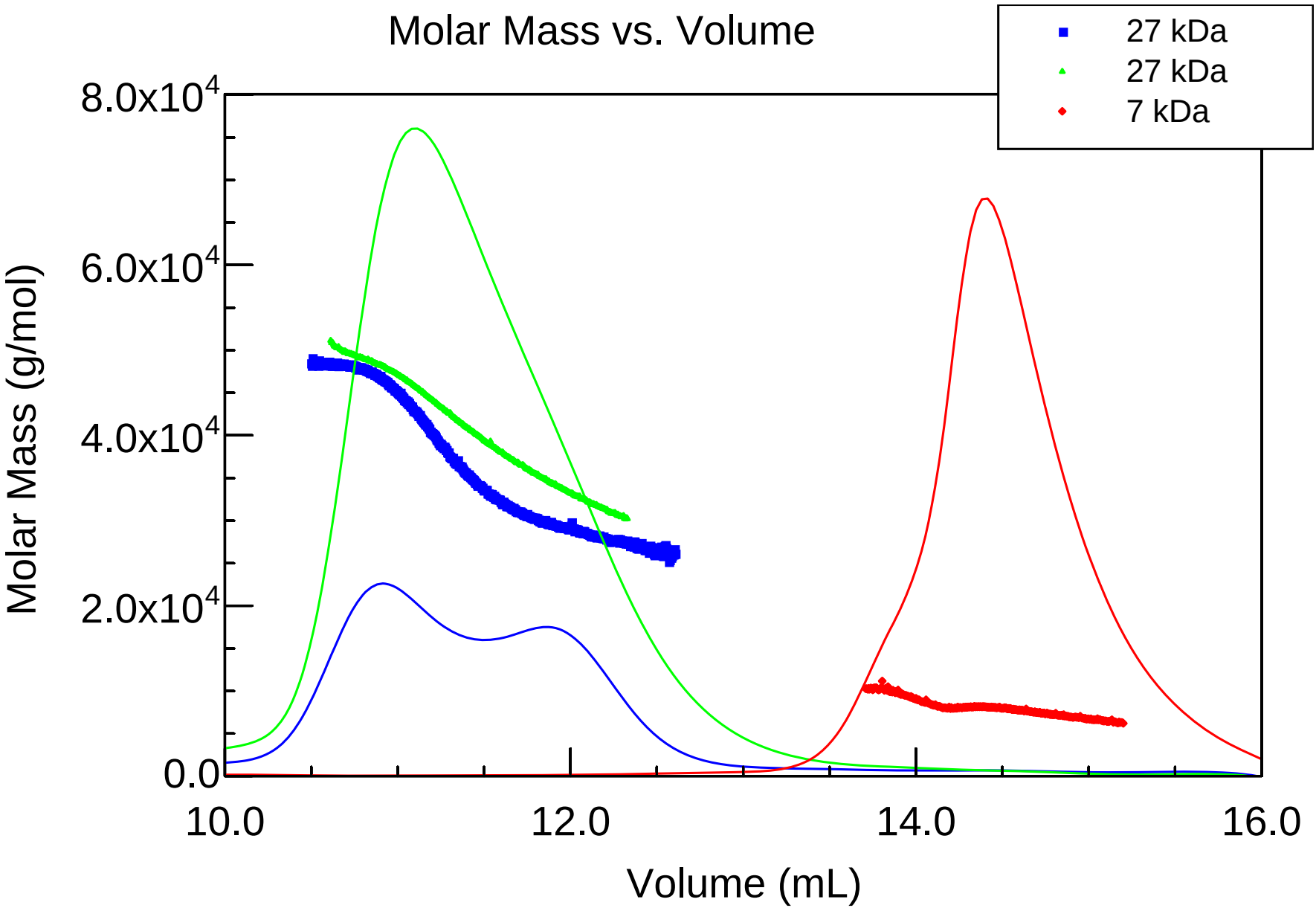
*ligand 7 kDa*

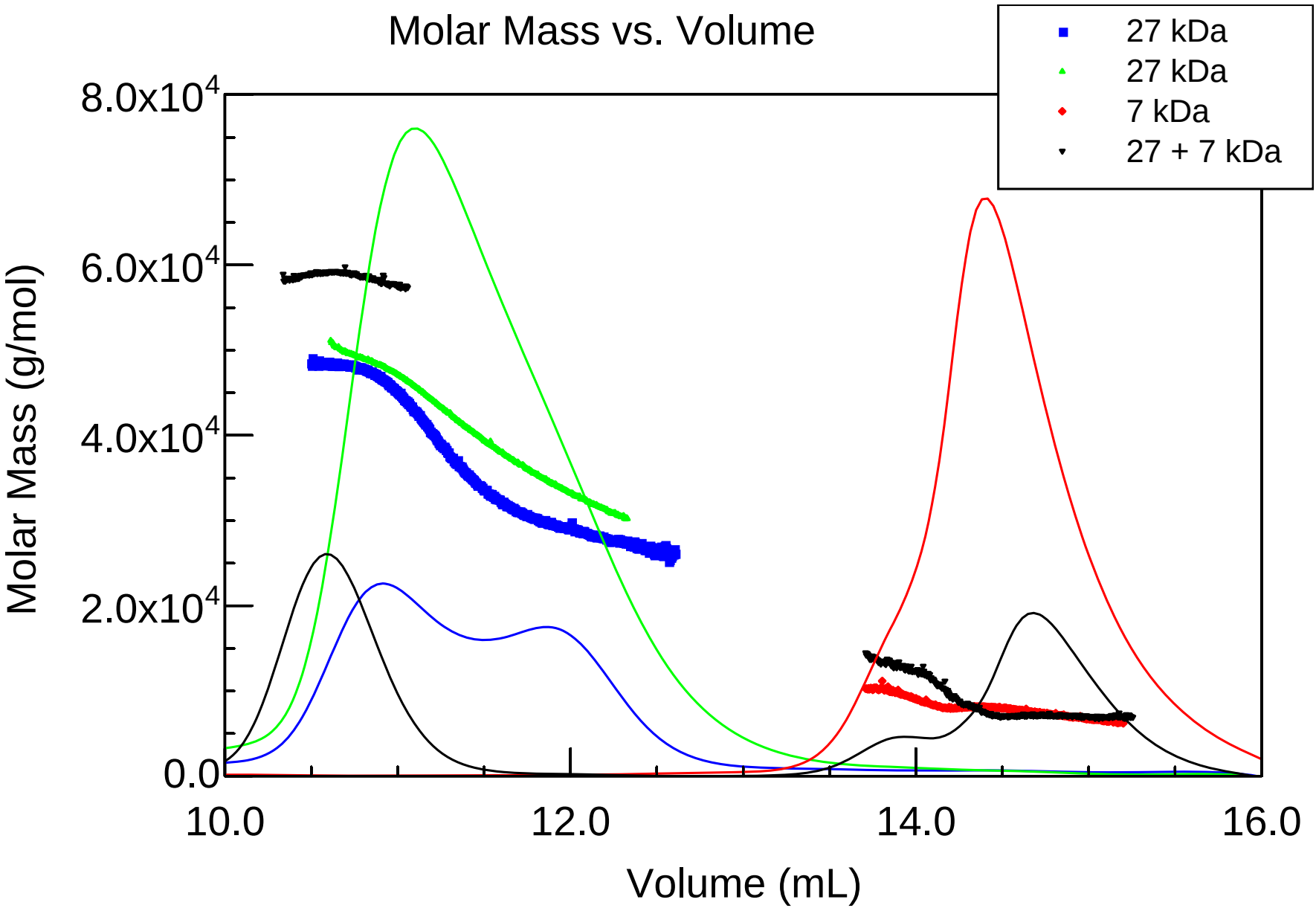
*Ligand binding shifts the protein into dimeric form*

# *Analysis of interacting proteins*

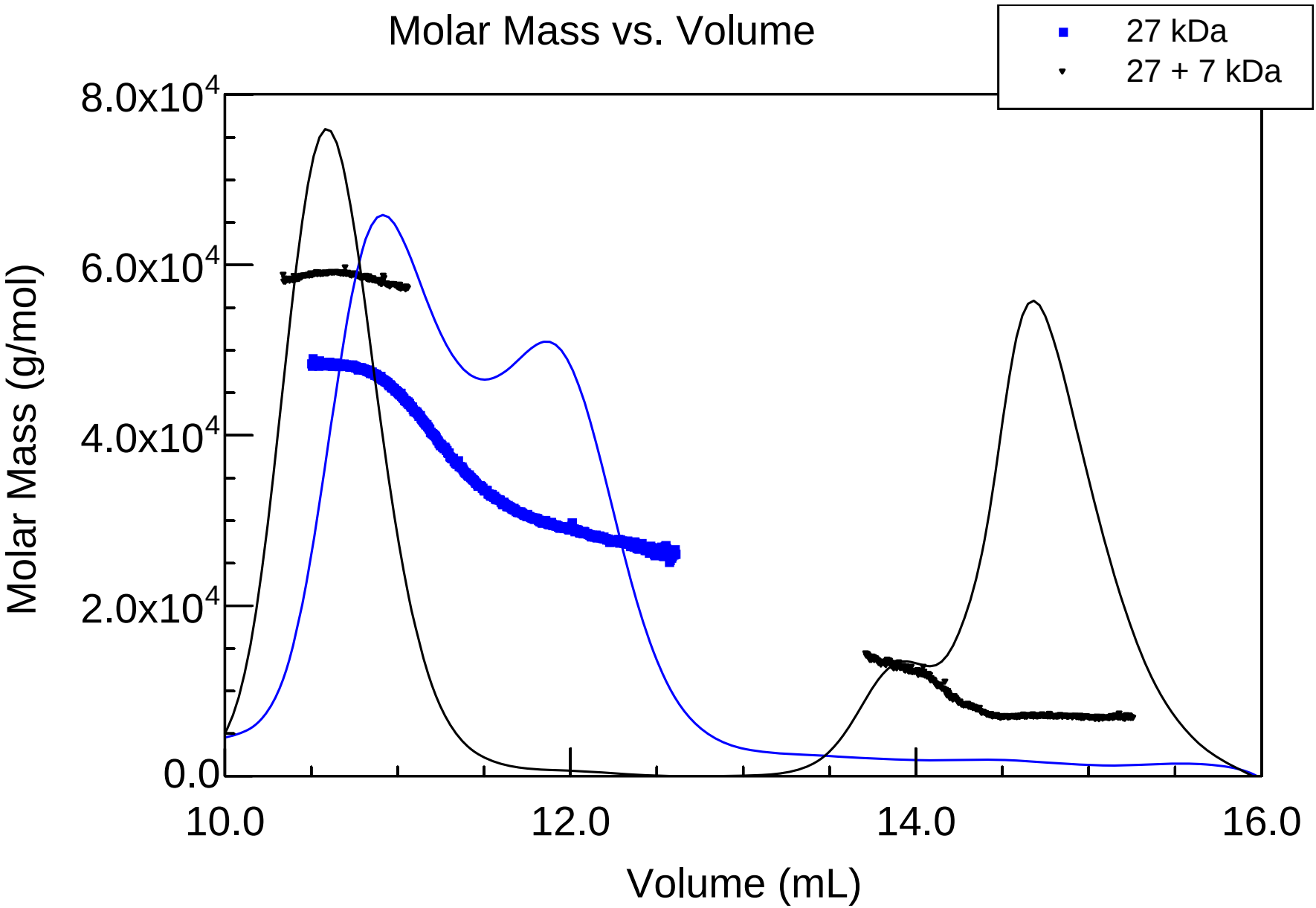



Molar Mass vs. Volume
27 kDa
27 kDa
8.0x10^4
6.0x10^4
4.0x10^4
2.0x10^4
0.0
Molar Mass (g/mol)
10.0
12.0
14.0
16.0
Volume (mL)


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

**Complex:** *(2*27)+7=61 kDa measured* ***MW=59 kDa***



# *Determination of the oligomeric state of modified protein*

*Data Analysis:*

*Use "three detector method"*

*Use ASTRA*

*(knowing the amount of non-polypeptide moiety bound)*

*use weight-average dn/dc value*

# Three Detector Method

*Yutaro Hayashi, Hideo Matsui and Toshio Takagi*

*Methods Enzymol 1989;172:514-28*

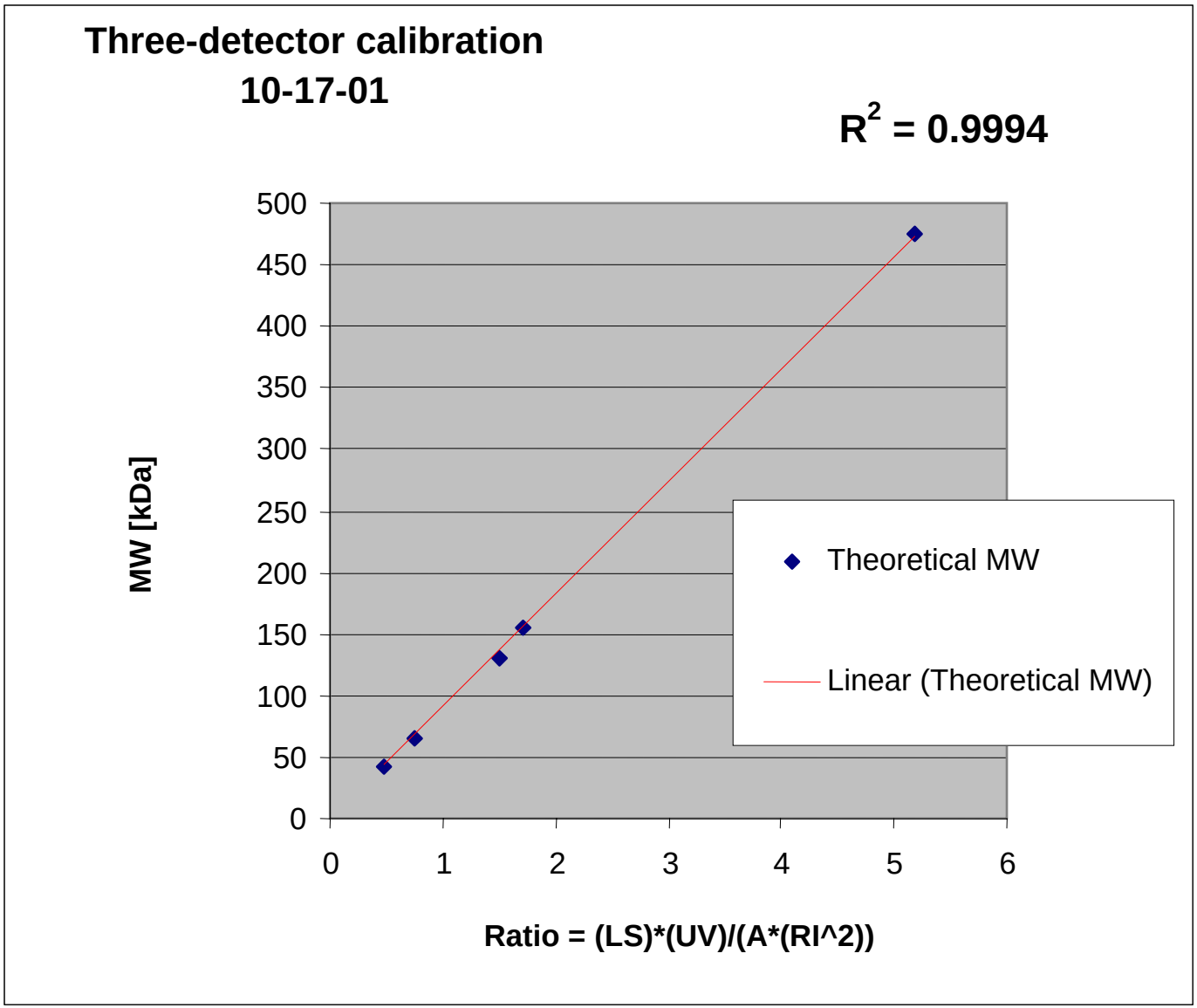
$$M_p = \frac{k^*(LS)(UV)}{\varepsilon(RI)^2}$$

*Jie Wen, Tsutomu Arakawa and John S. Philo*

*Anal Biochem 1996 Sep 5;240(2):155-66*

# MWp = 91.39 x [(LS)*(UV)/(A*(RI^2))]

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



# *Determination of the oligomeric state of detergent solubilized membrane protein*

*Data Analysis:*

*Use "three detector method"*

*Use ASTRA*

*use "corrected" dn/dc value as described by Habayashi (scaled RI signal such that it represents contribution only from polypeptide)*

# *Determination of the oligomeric state of detergent solubilized protein*

*Example:*

*protein* *47 kDa* *well characterized porin*

*detergent*

*dodecyl maltoside (C12M) MW = 511 g/mol*

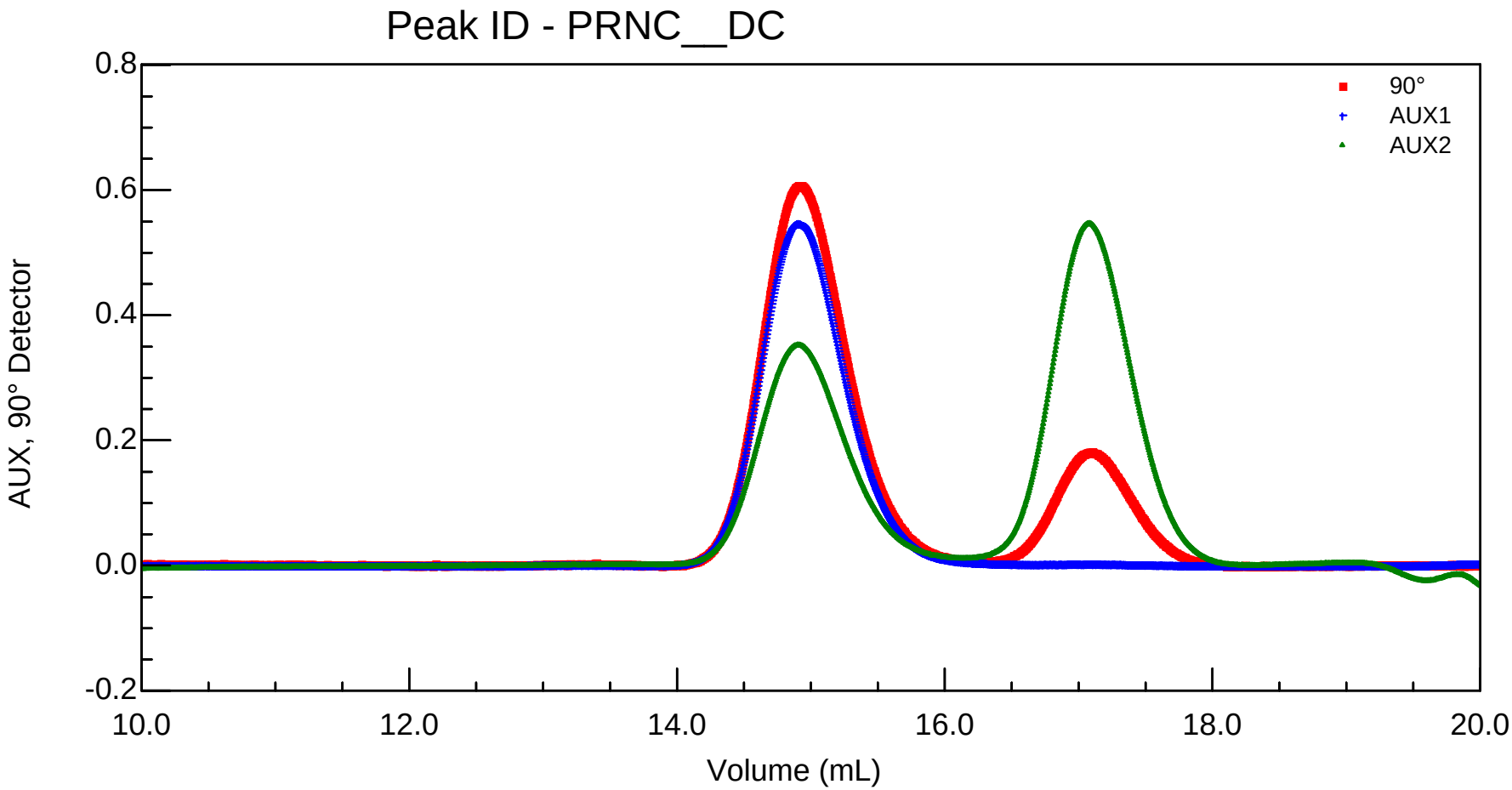
*0.5g/L i.e. 0.05%*

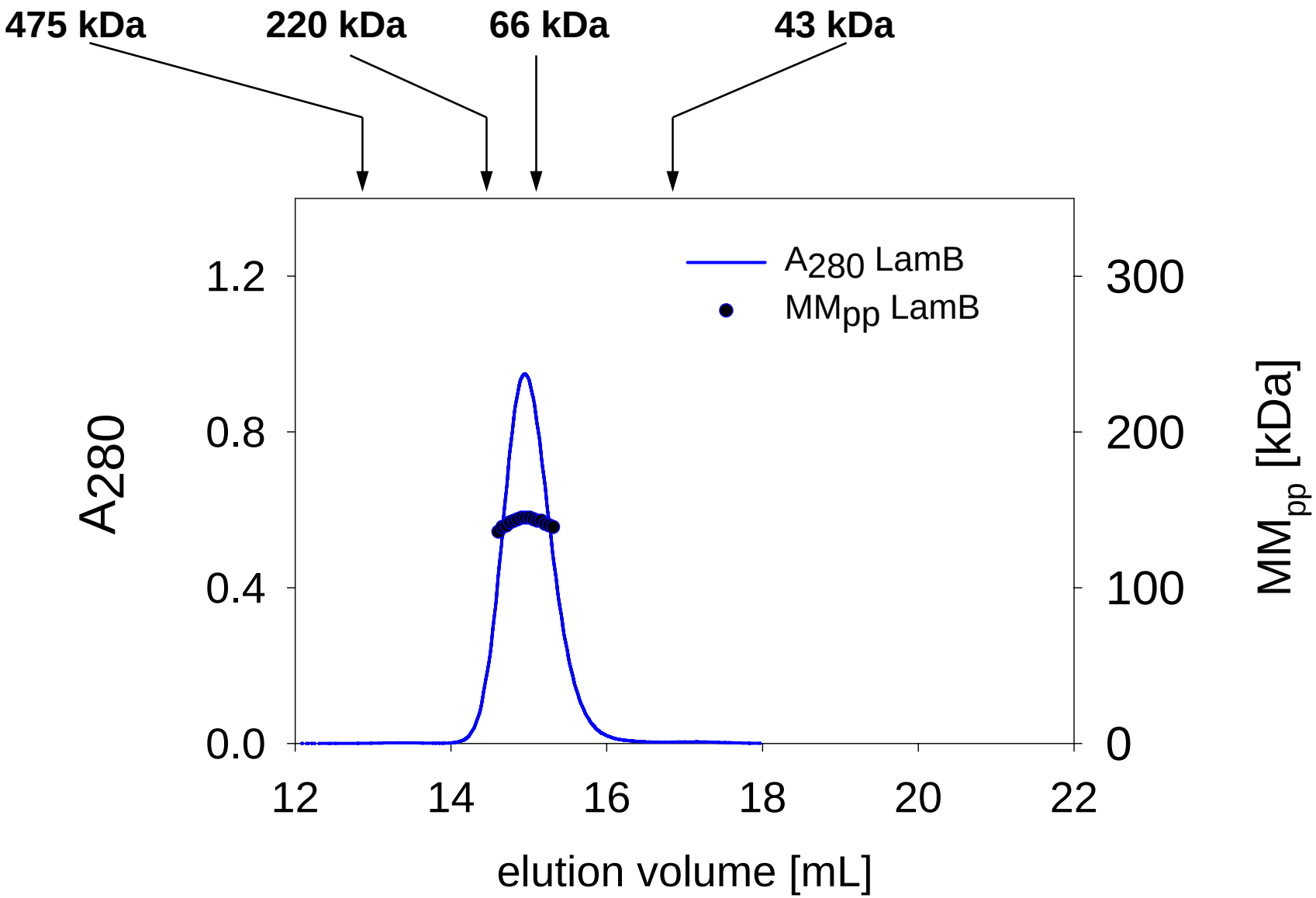
*CMC = 0.008% micelle size 50-70 kDa*

*LS @ 90 degree*

*RI*

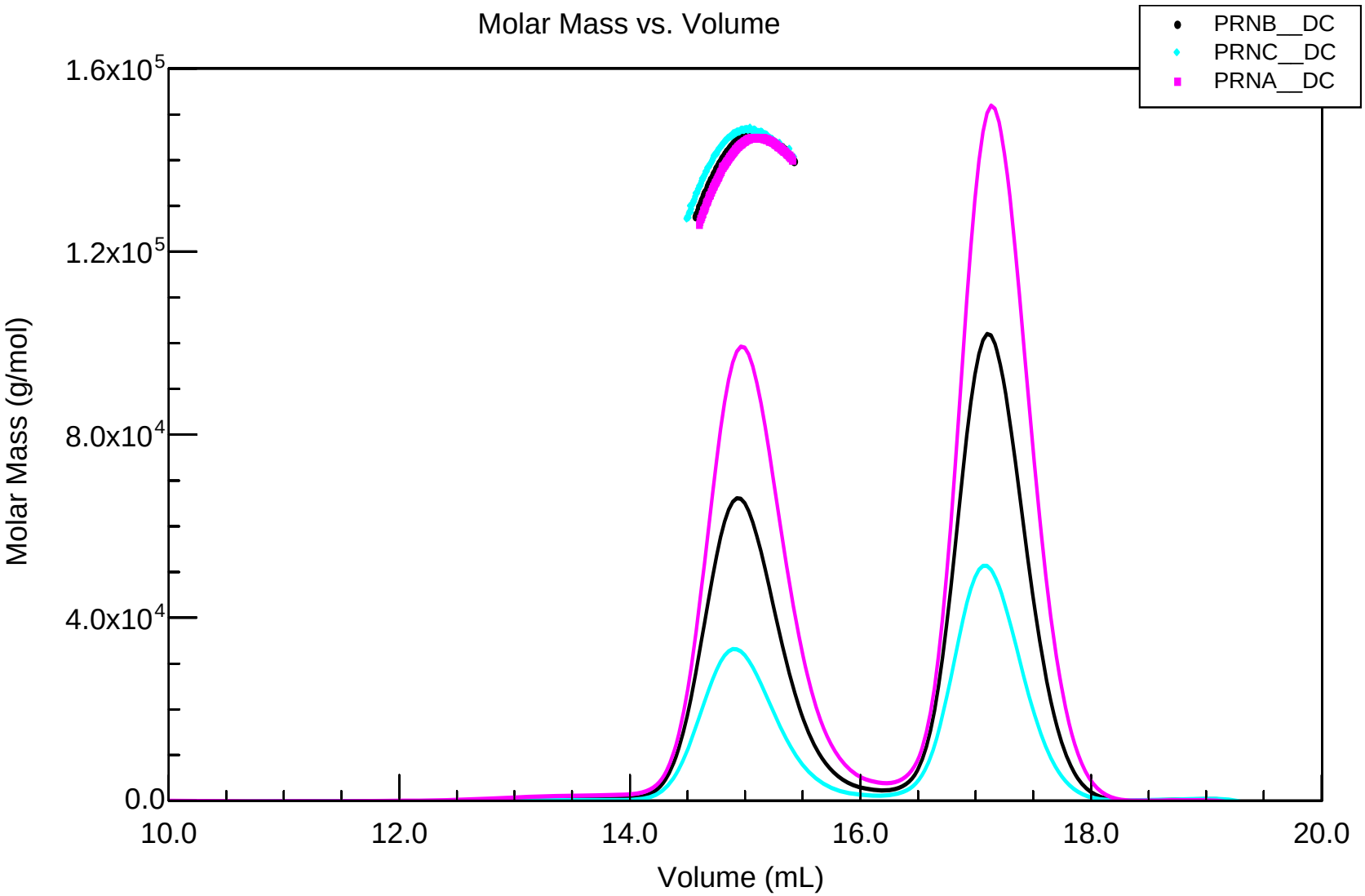
*UV @ 280 nm*




475 kDa
220 kDa
66 kDa
43 kDa
A280 LamB
MMpp LamB
A280
MMpp [kDa]
elution volume [mL]
0.0
0.4
0.8
1.2
0
100
200
300
12
14
16
18
20
22

*porin  monomer = 47 kDa*

*MW = 149 ± 3  kDa  trimer*



# *Determination of the oligomeric state of detergent solubilized protein*

*Example:*

*protein* *33 kDa*

*Detergent*

*dodecyl maltoside (C12M) MW = 511 g/mol*

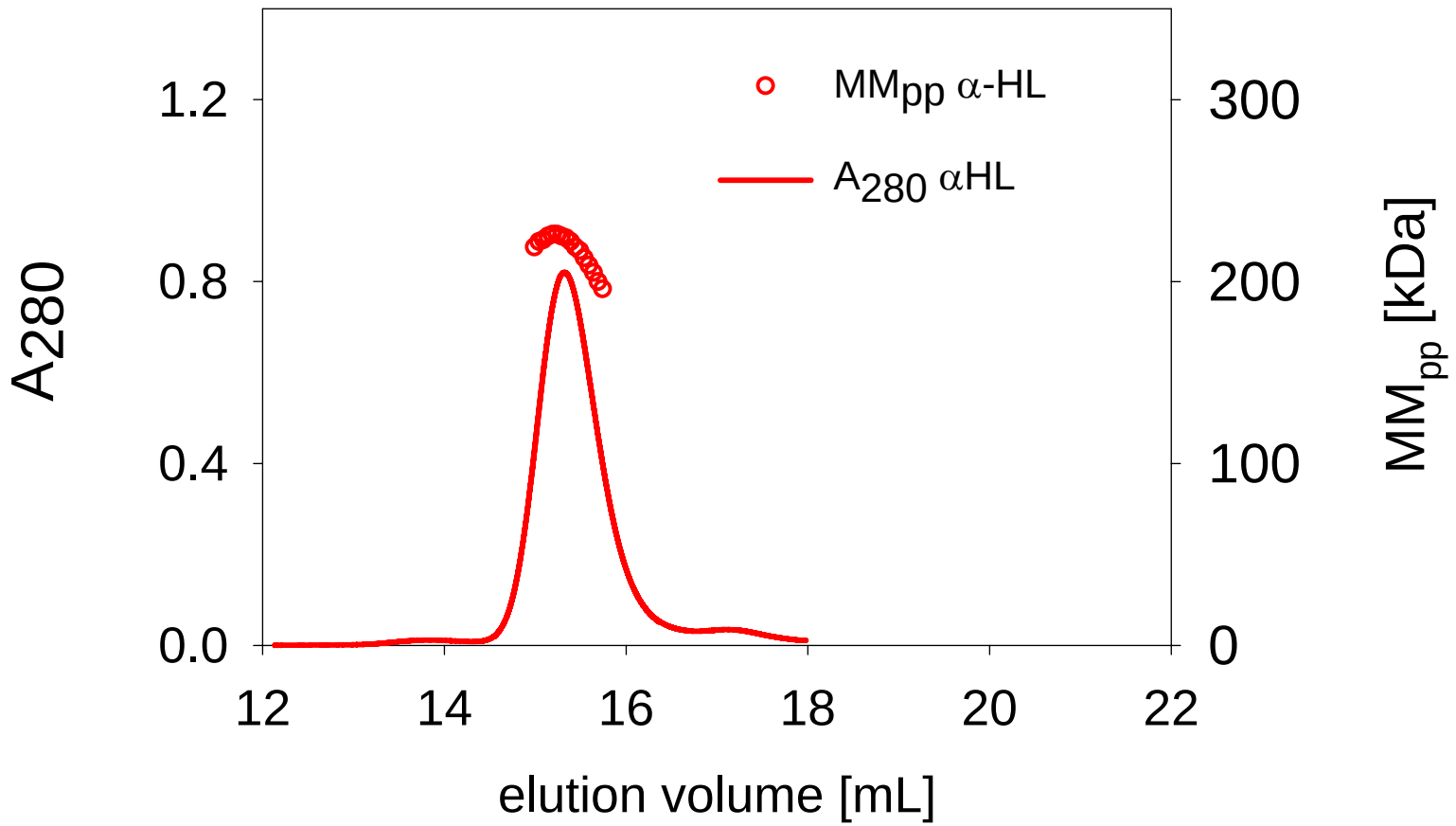
*n-Dodecyl-β-D-Maltoside*

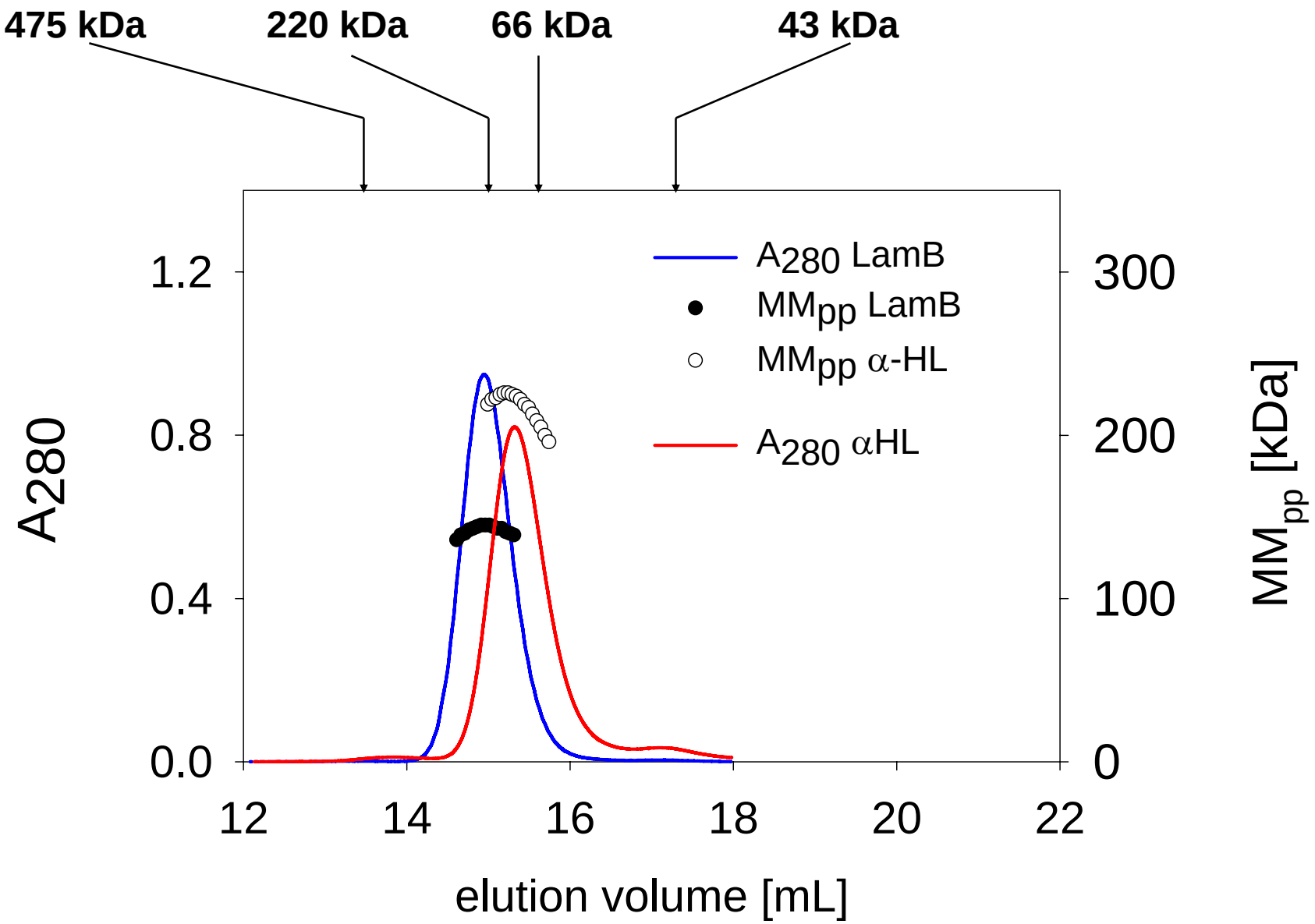
*0.5g/L i.e. 0.05%*

*CMC = 0.008% micelle size 50-70 kDa*

*monomer = 33 kDa*

*MW = 225 ± 13 kDa heptamer*




475 kDa
220 kDa
66 kDa
43 kDa
A280 LamB
MMpp LamB
MMpp α-HL
A280 αHL
A280
MMpp [kDa]
elution volume [mL]
0.0
0.4
0.8
1.2
0
100
200
300
12
14
16
18
20
22

# Three Detector Method

*Yutaro Hayashi, Hideo Matsui and Toshio Takagi*

*Methods Enzymol 1989;172:514-28*

*allows determination of mass of detergent/lipids bound to a polypeptide*

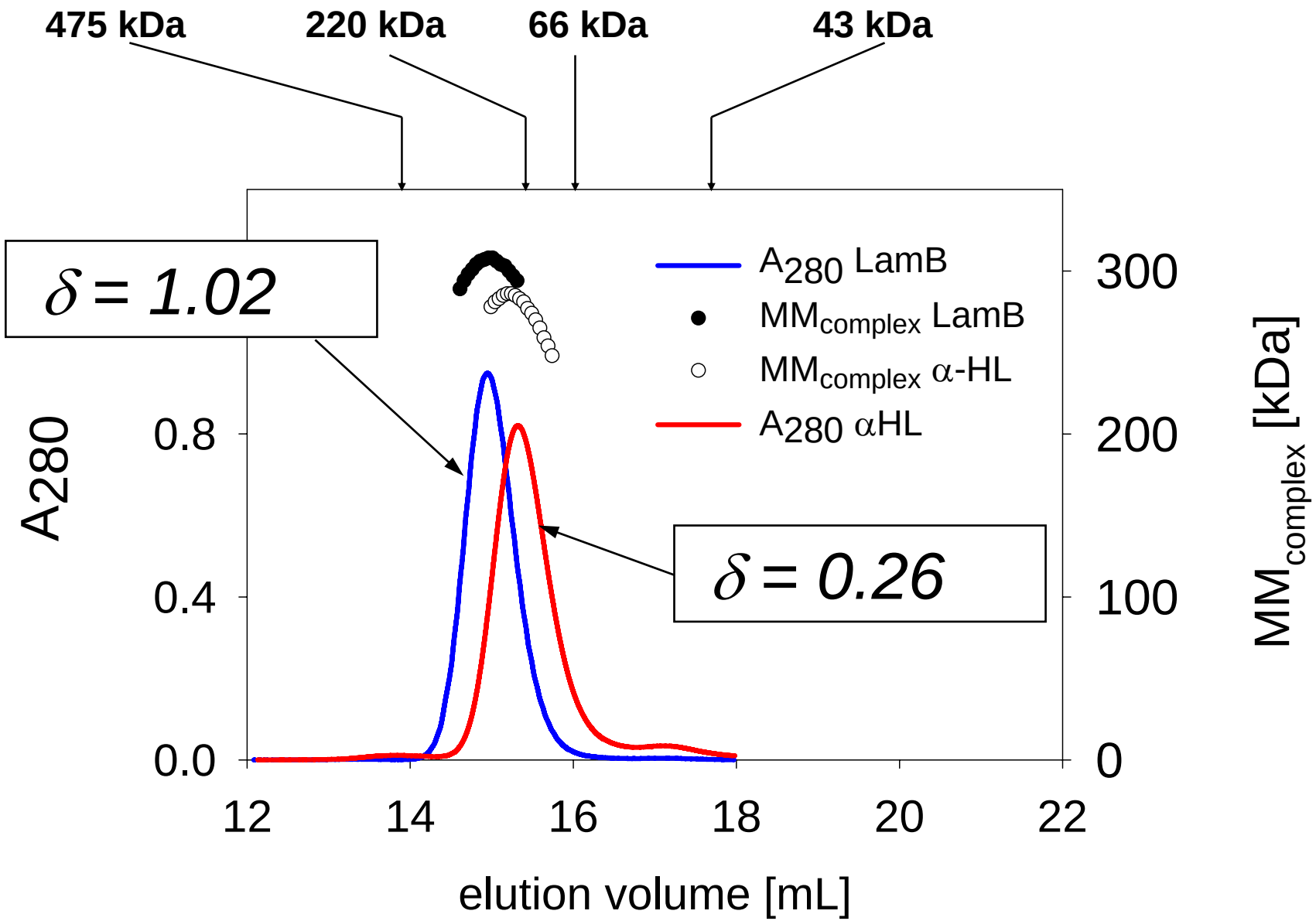
$$\left(\frac{dn}{dc}\right)_{app} = k_2 A \frac{(RI)}{(UV)}$$

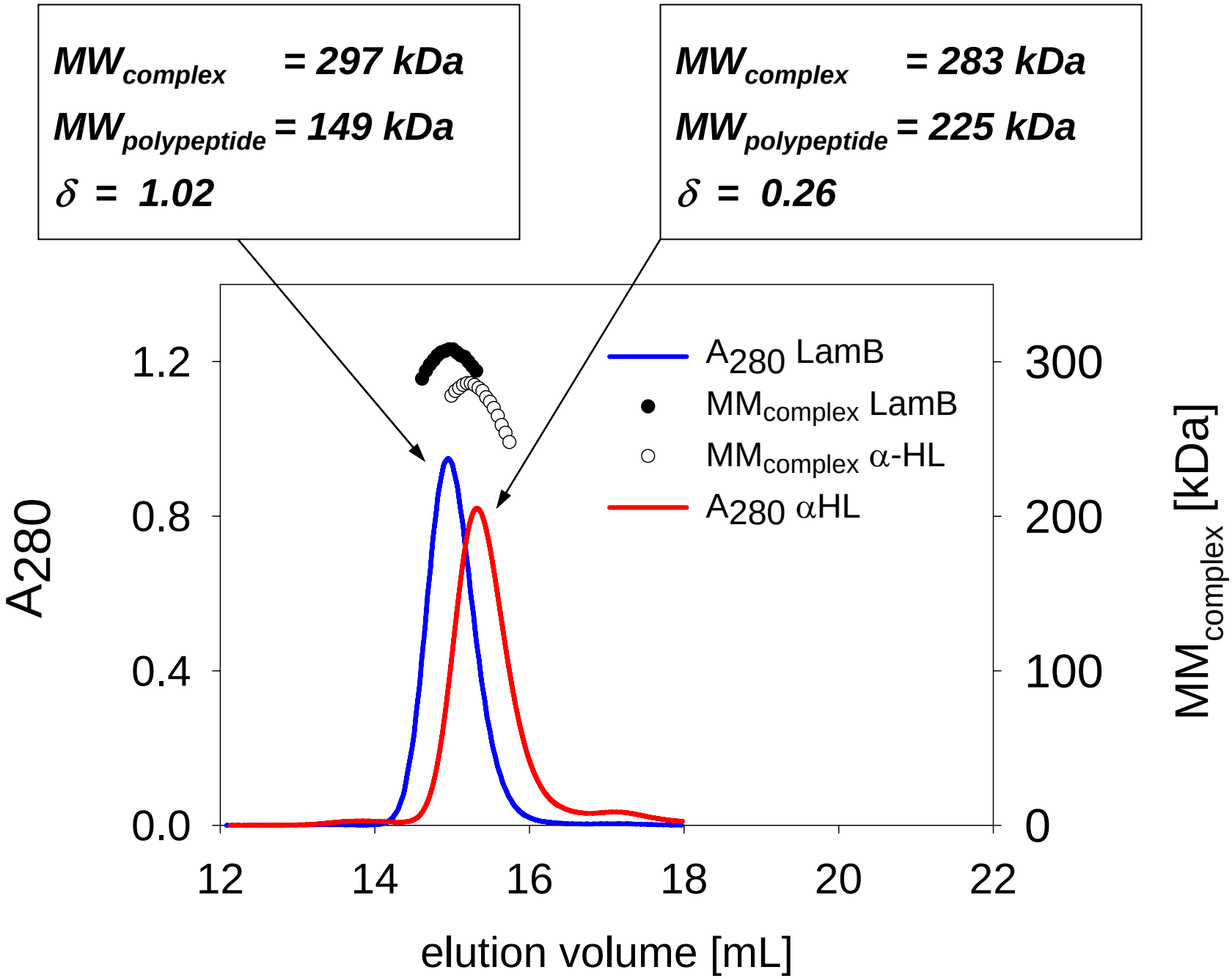
$$\left(\frac{dn}{dc}\right)_{app} = \left(\frac{dn}{dc}\right)_{pp} + \delta \left(\frac{dn}{dc}\right)_{d+l} = K' \frac{(RI)}{\varepsilon(UV)}$$

$\delta$ *is mass of detergent and/or lipids per 1 gram of polypeptide*

*Assumption : detergent does not produce any signal in UV*

$\delta$ is mass of detergent and/or lipids per 1 gram of polypeptide




$MW_{complex}$ = 297 kDa
$MW_{polypeptide}$ = 149 kDa
$\delta$ = 1.02
$MW_{complex}$ = 283 kDa
$MW_{polypeptide}$ = 225 kDa
$\delta$ = 0.26
$A_{280}$ LamB
$MM_{complex}$ LamB
$MM_{complex}$ α-HL
$A_{280}$ αHL
A280
0.0
0.4
0.8
1.2
0
100
200
300
$MM_{complex}$ [kDa]
12
14
16
18
20
22
elution volume [mL]

# *Conclusions*

# *SEC coupled with Static LS/RI/UV*

- 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 detection and characterization of non-interacting and interacting systems
- SEC/LS/UV/RI analysis can determine oligomeric state of modified proteins including detergent solubilized membrane proteins

*Ken Williams*

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

*NIH*

*Users of SEC/LS Service*