Analysis of Protein Complexes by Size Exclusion Chromatography Coupled with Light Scattering

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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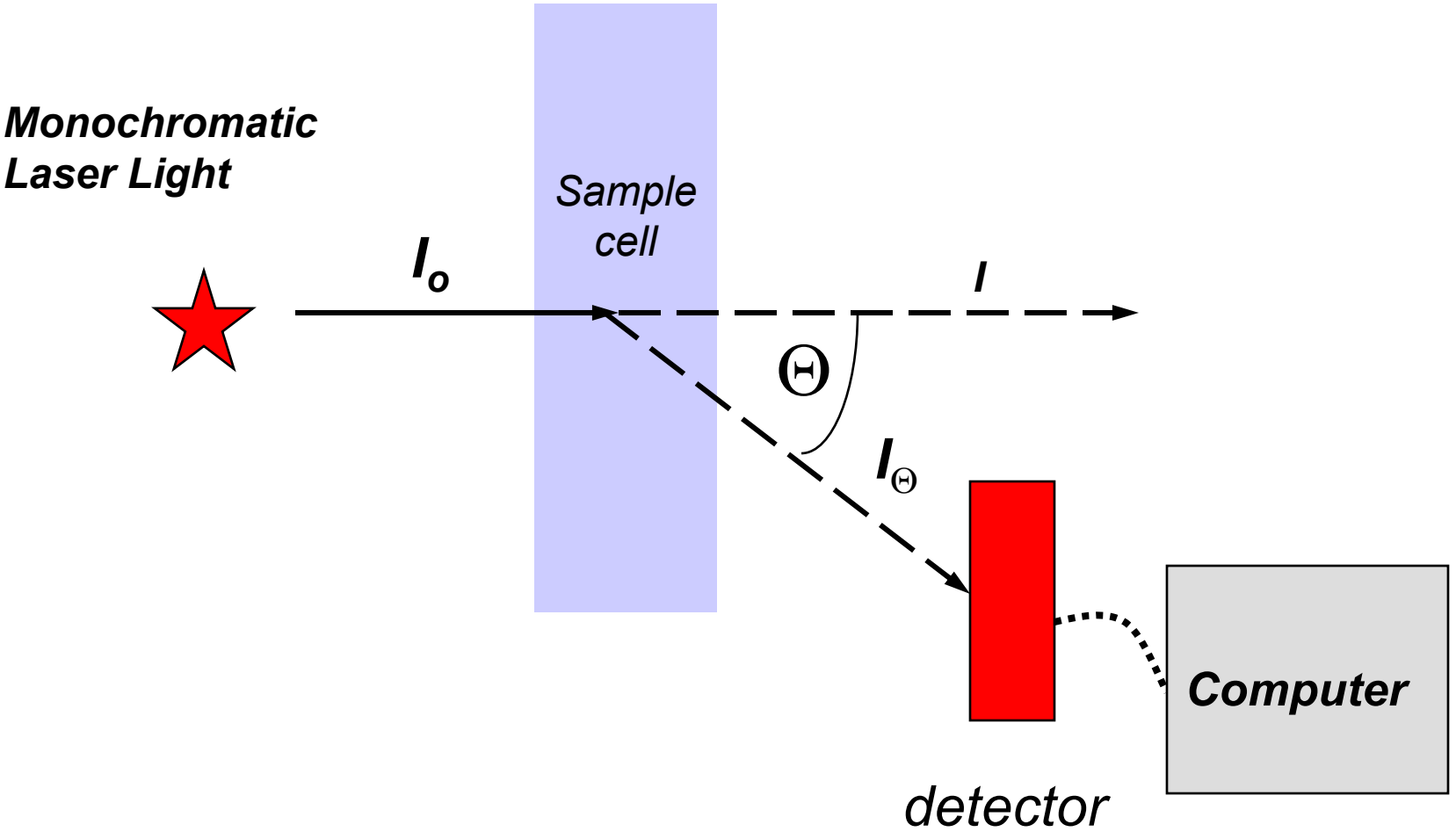
Light Scattering Experiments



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
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

at low concentrations c < 0.1 mg/mL

$$2A_2cMw<<1$$

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

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

expansion of P(Θ) to the first order gives

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

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

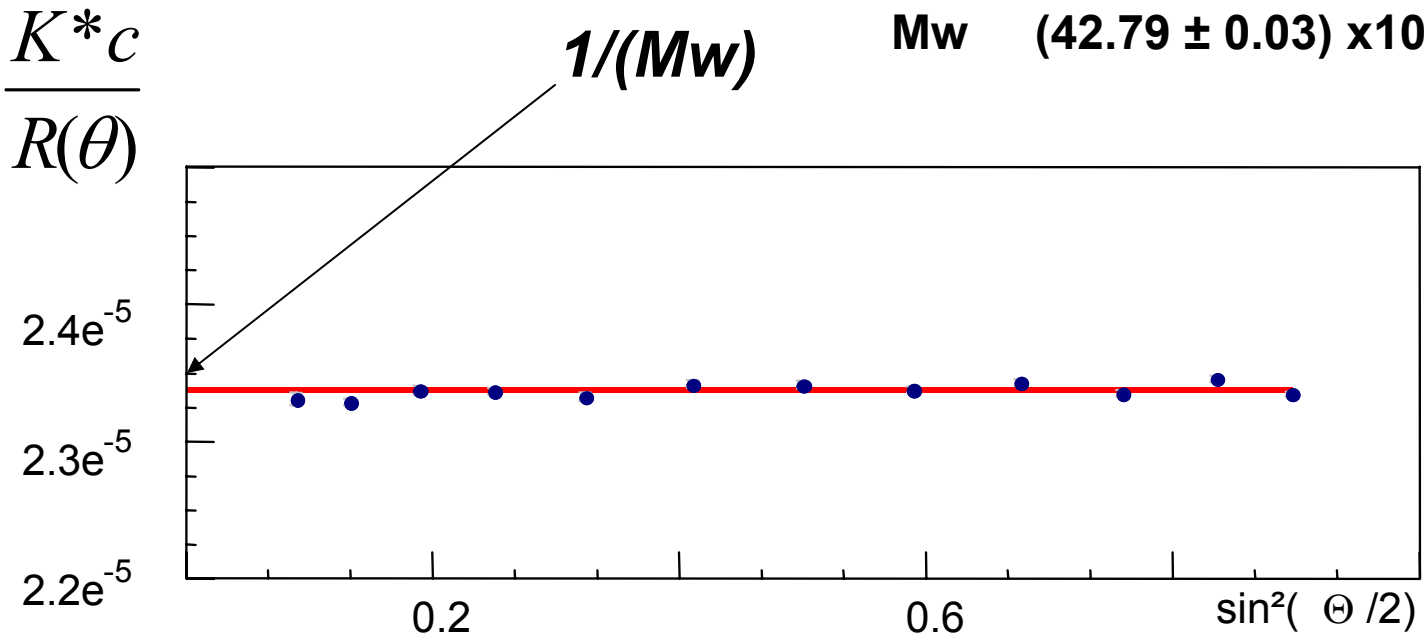
construct a plot of

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

From intercept ⟶ Derived MW

Zimm Plot Ovalbumin (43 kDa)

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



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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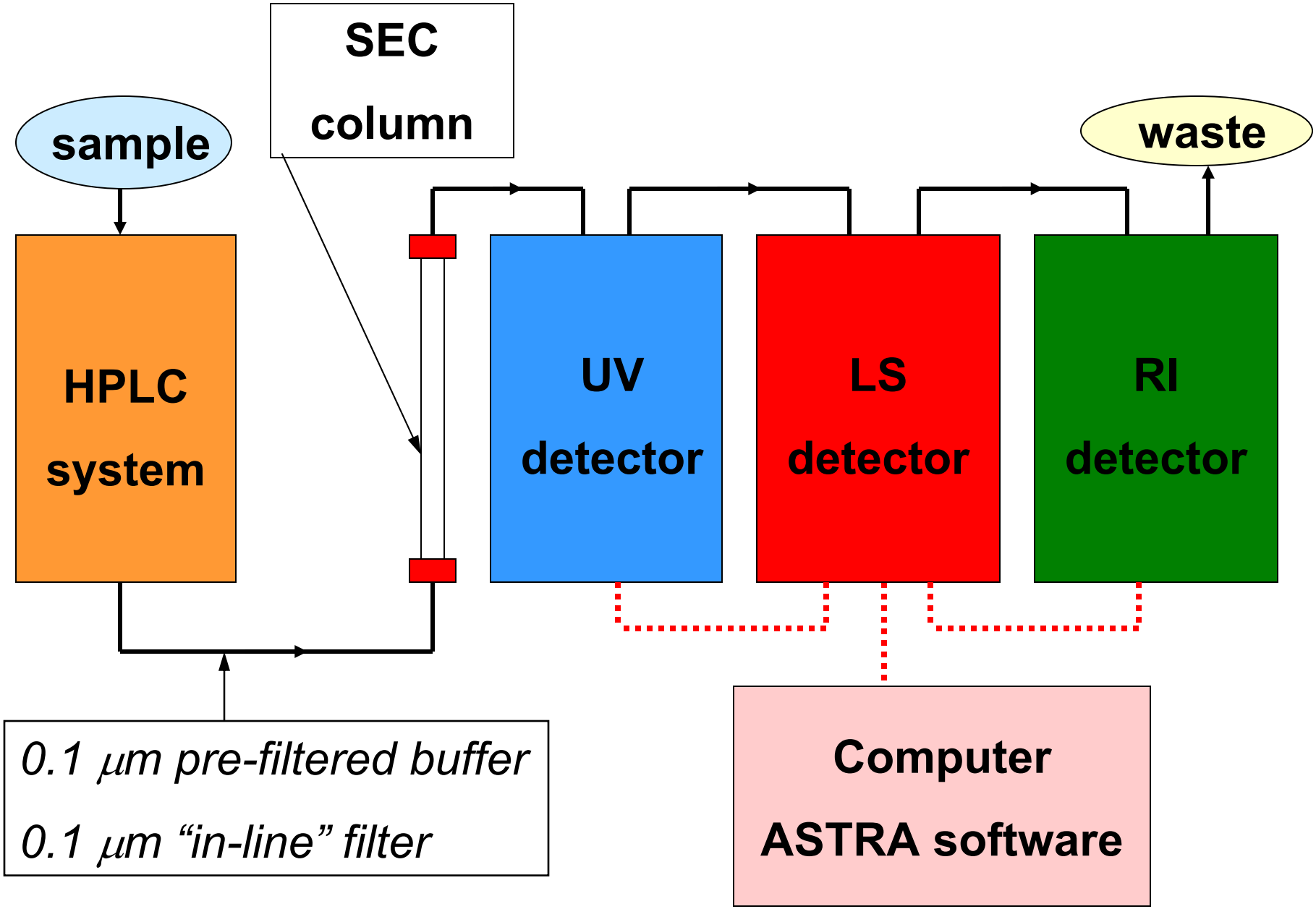
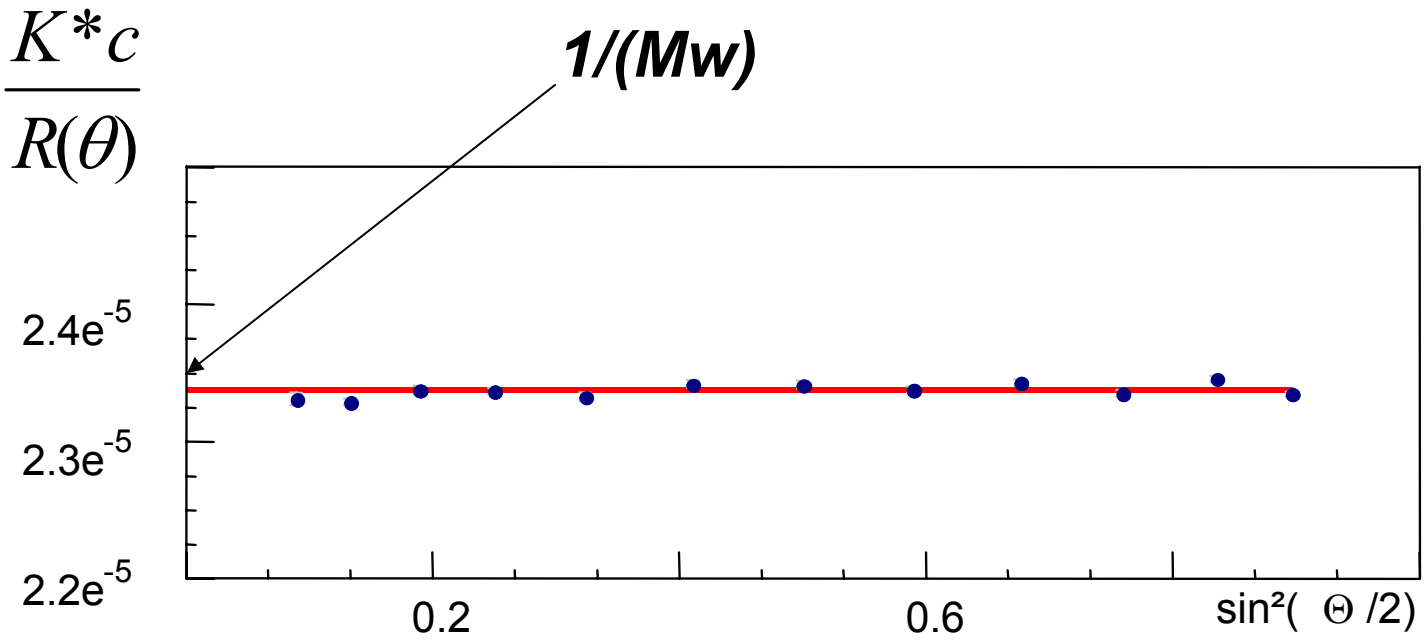

SEC
column
sample
waste
HPLC
system
UV
detector
LS
detector
RI
detector
0.1 μm pre-filtered buffer
0.1 μm "in-line" filter
Computer
ASTRA software

Zimm Plot Ovalbumin (43 kDa)

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

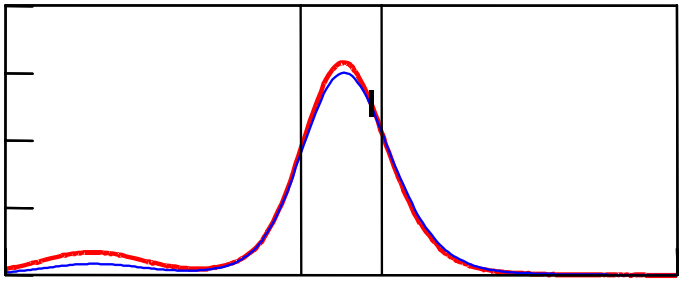


Volume : 16.300 mL

Conc. : (0.173 ± 0.000) mg/mL

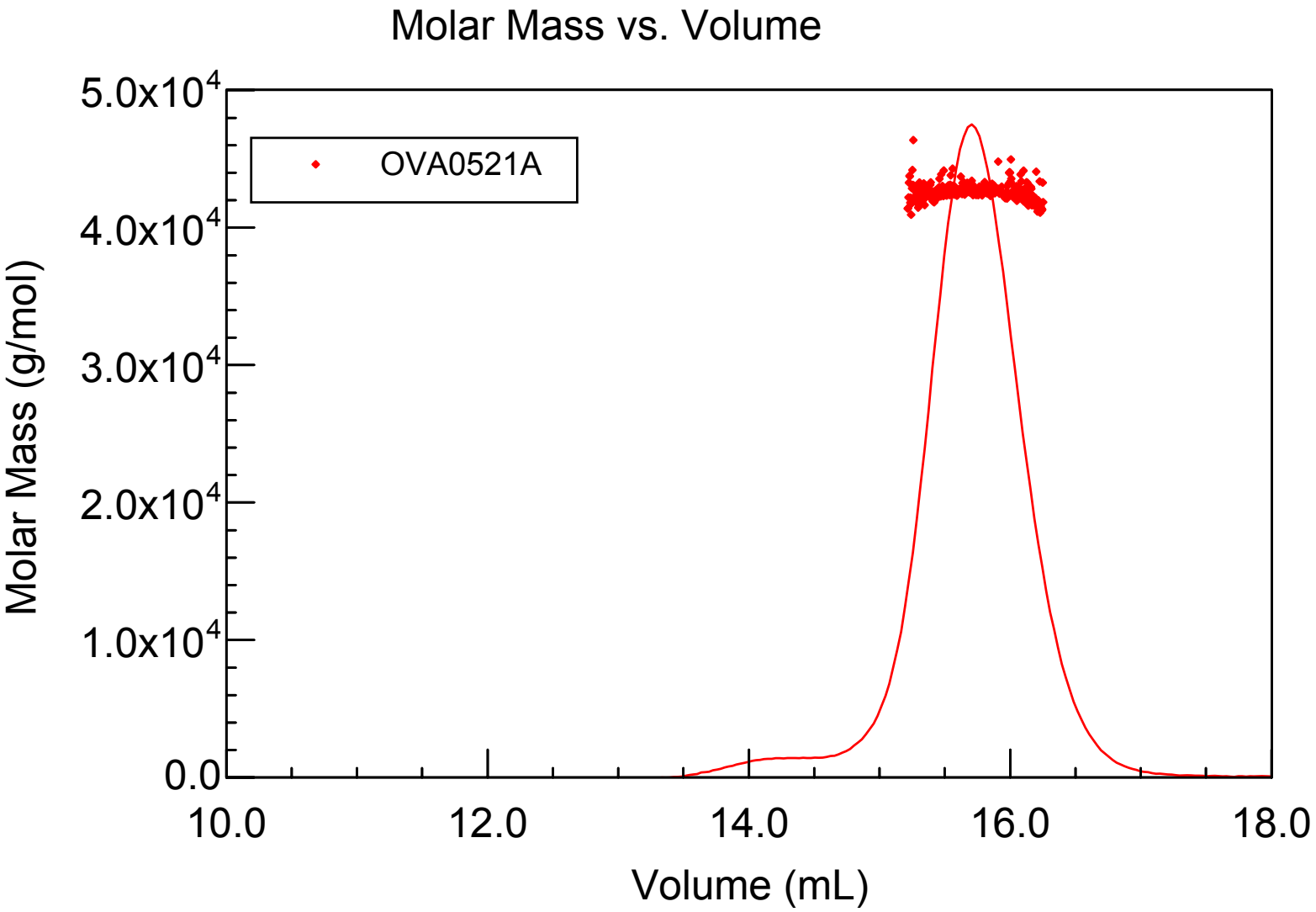
Mw (42.79 ± 0.03) x10^3 g/mol

Radius : 0.0 ± 0.0nm



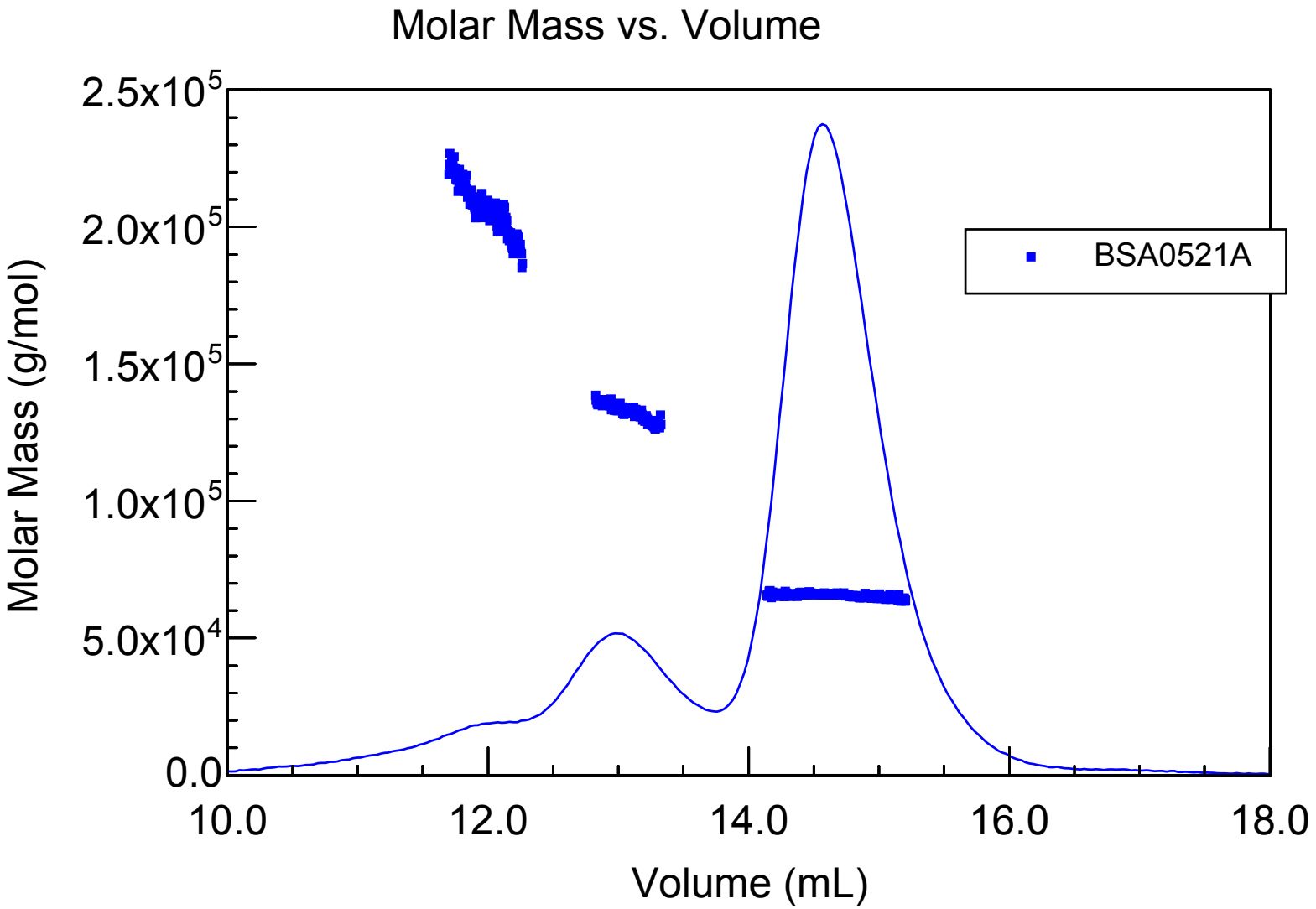
Molar Mass Distribution Plot

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			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

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Applications of SEC/LS to study protein complexes

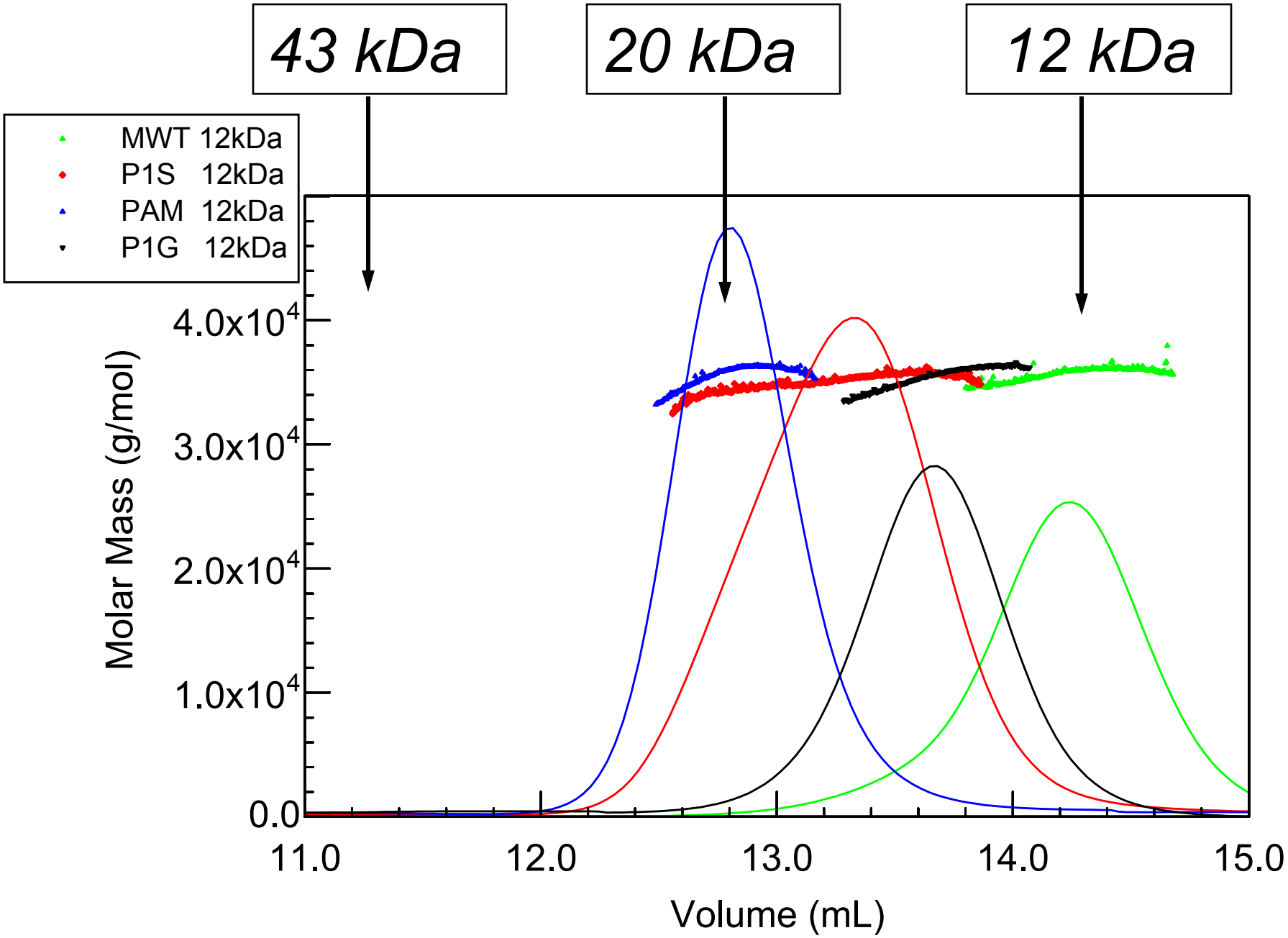
- *Determination of the oligomeric state of mutant vs. wild type protein*
- *Mixtures of non-interacting proteins*
- *Mixtures of interacting protein- detection of ligand driven protein complexes*
- *Determination of oligomeric state of glycosylated proteins*
- *Determination of oligomeric state of membrane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

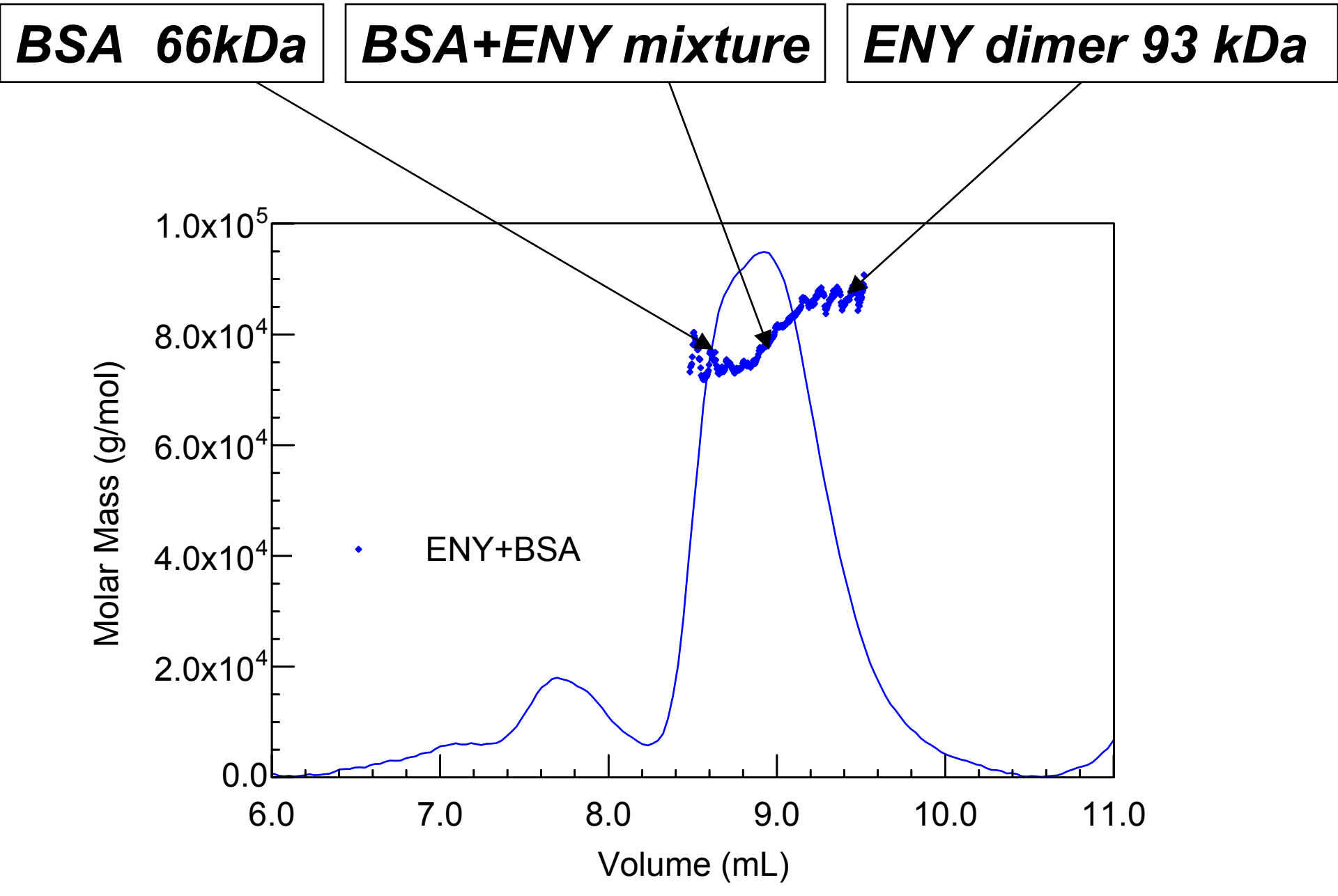
Mixtures of non-interacting proteins

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

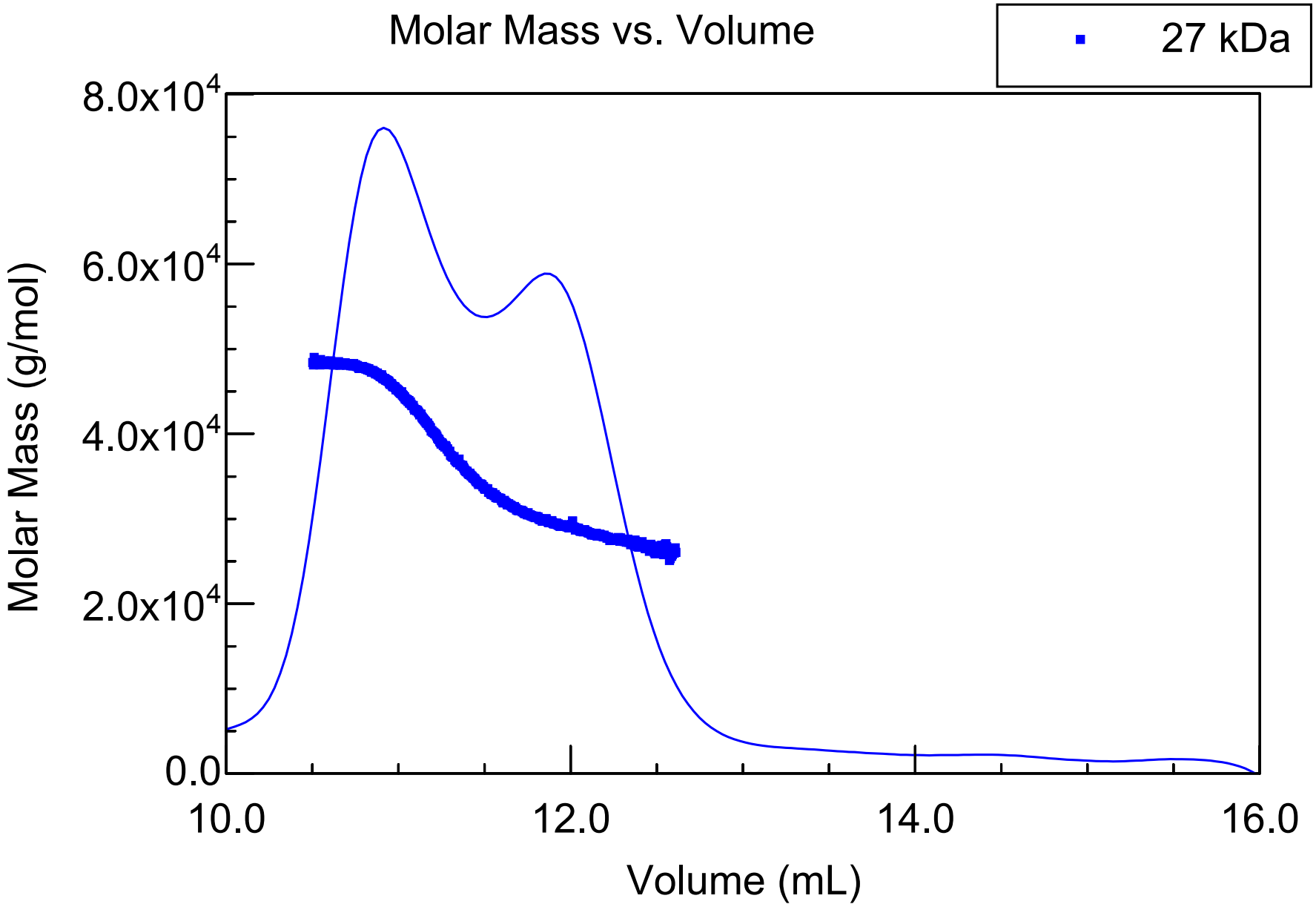
Example:

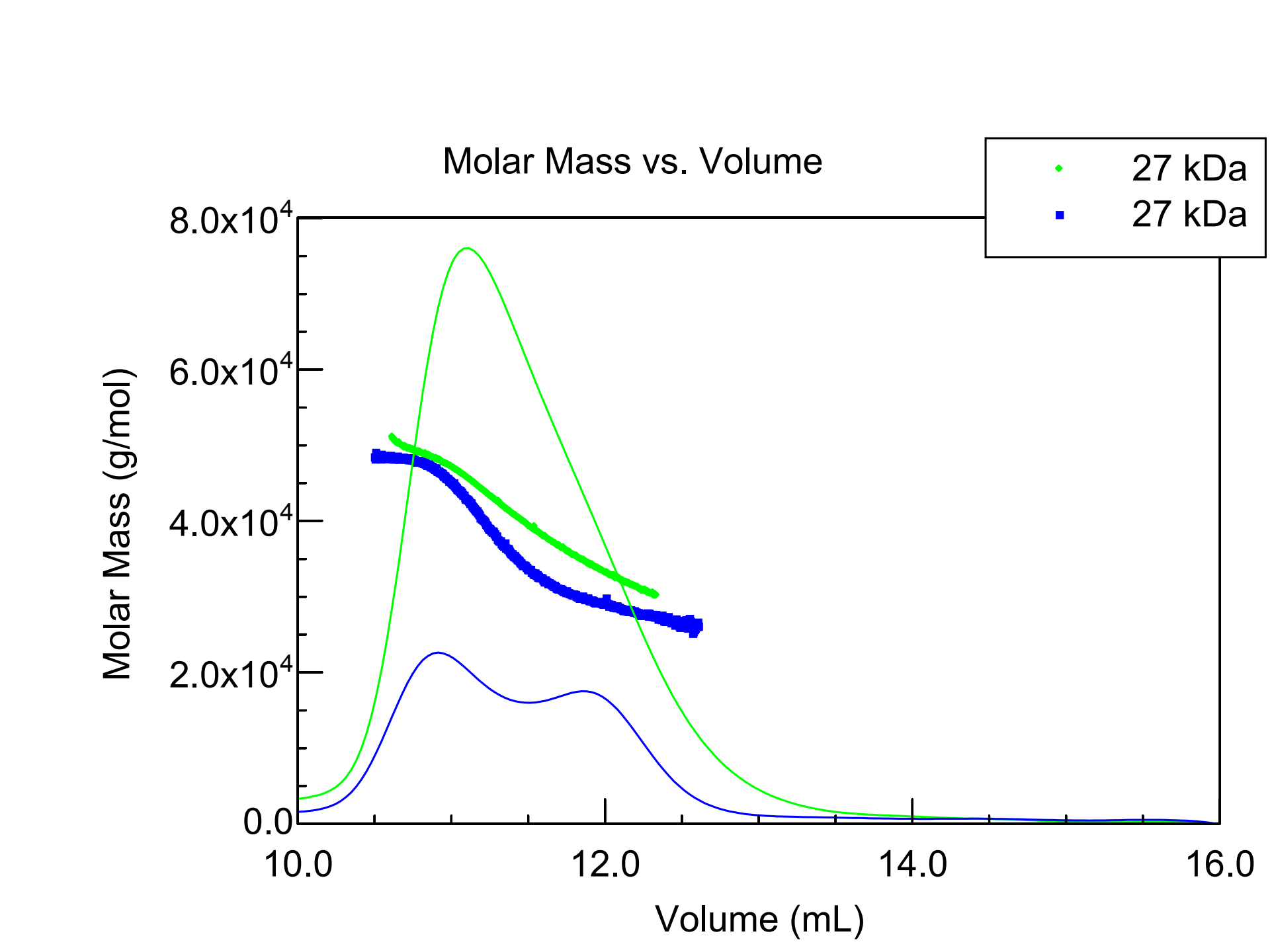
protein 27 kDa (protein exists as a mixture of monomer and dimer)

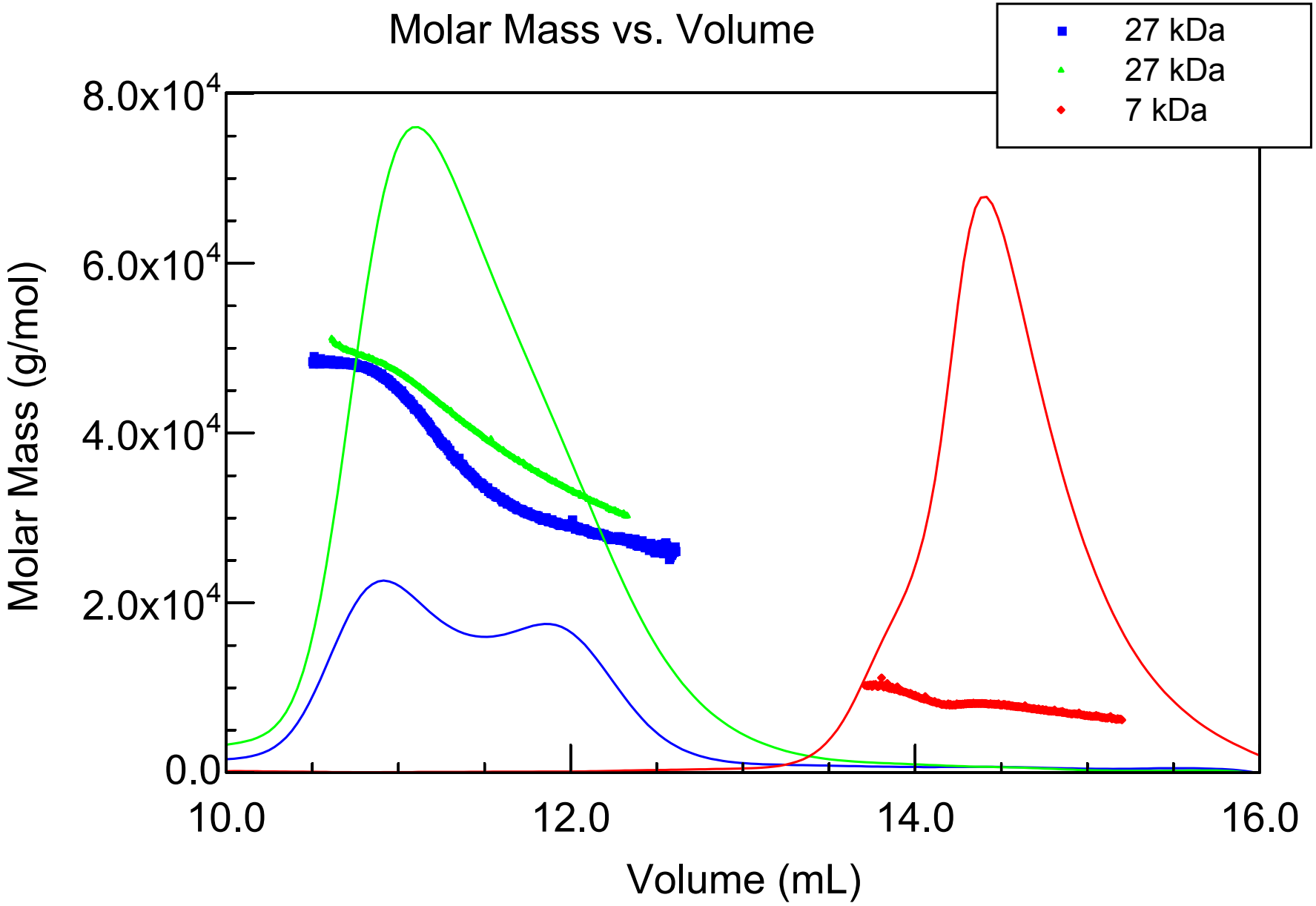
ligand 7 kDa

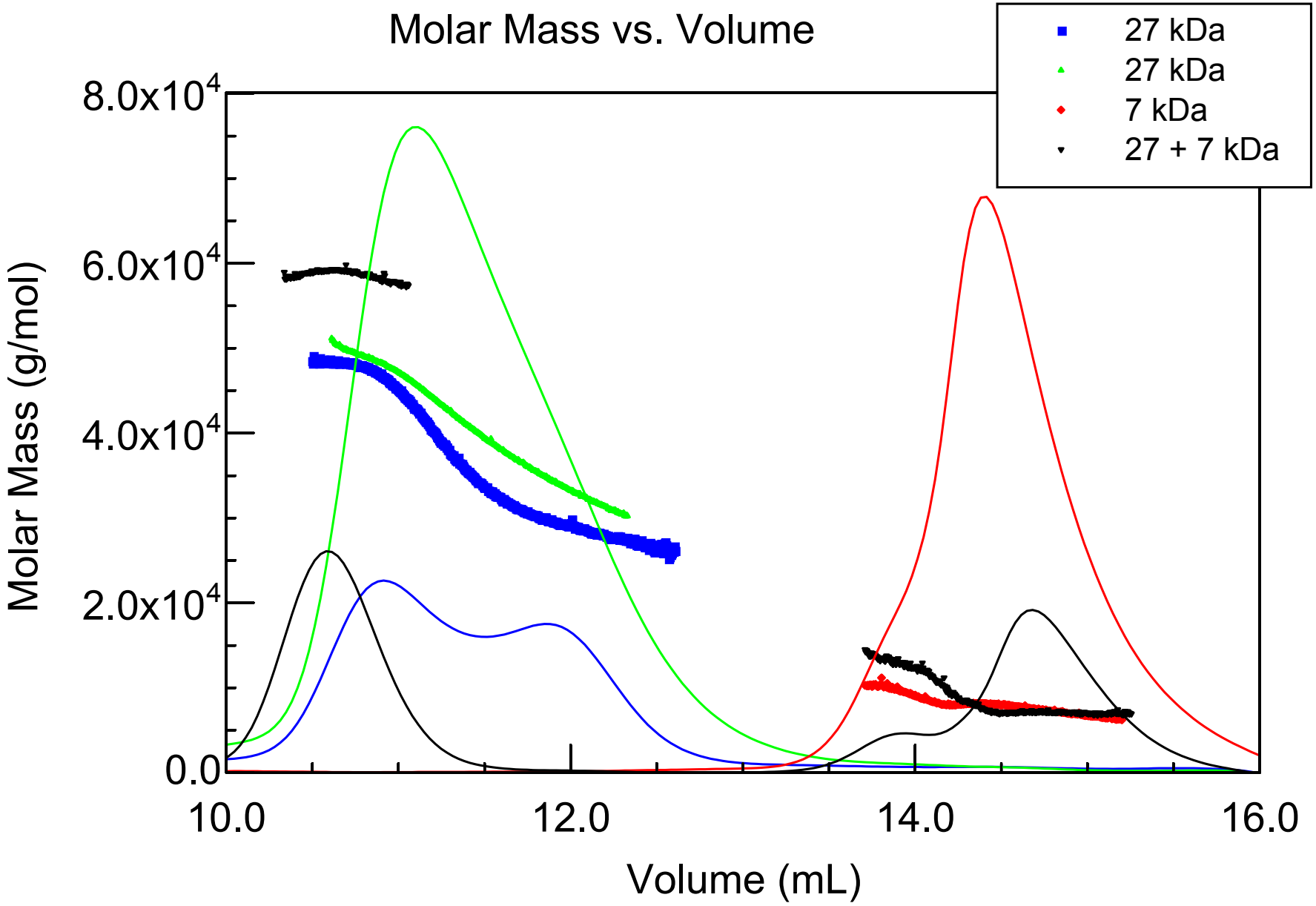
Ligand binding shifts the protein into dimeric form

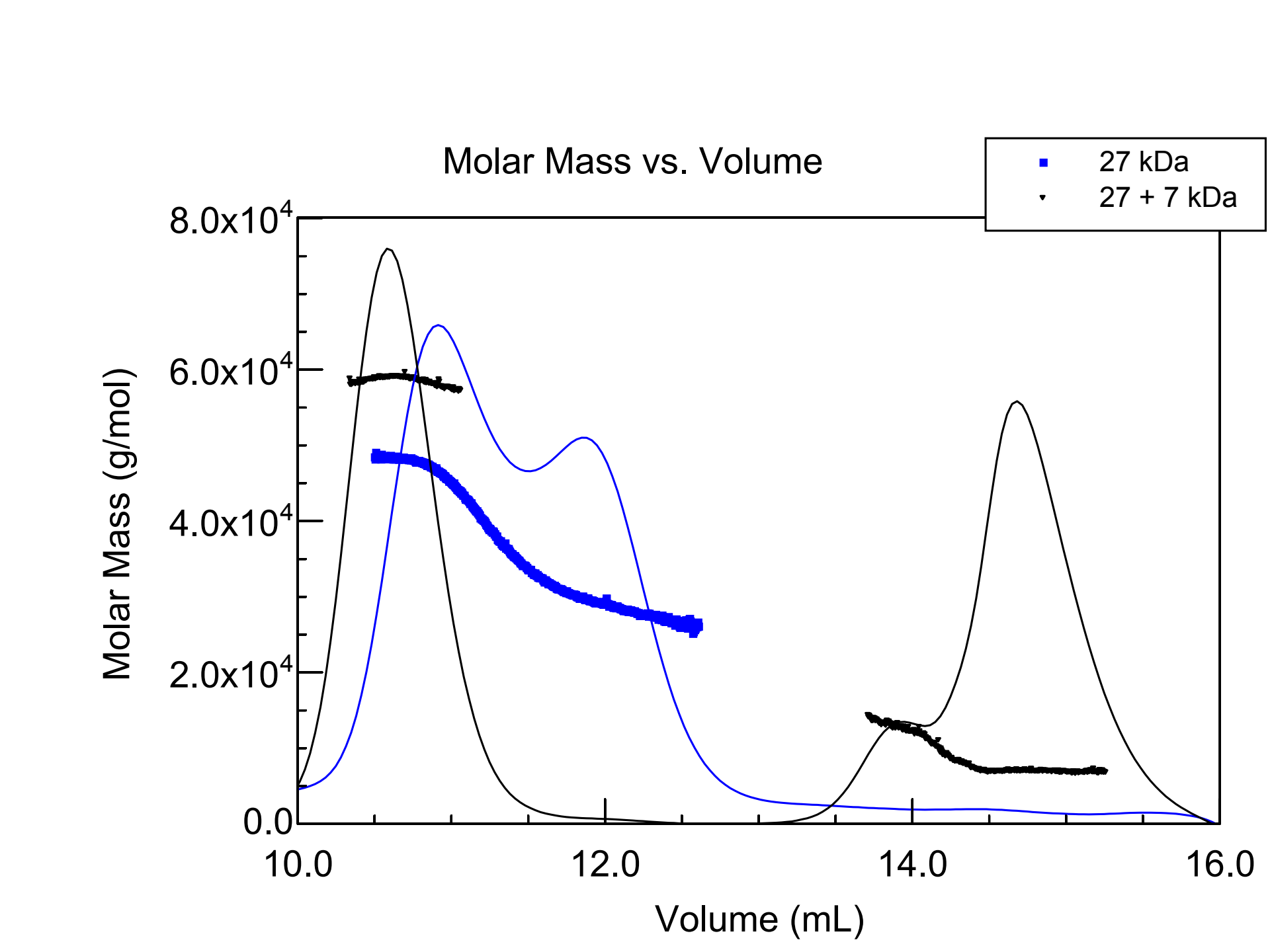
Analysis of interacting proteins




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)


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 glycosylated protein

Data Analysis:

Use "three detector method"

Use ASTRA

(knowing the amount of sugars bound)

use weight-average dn/dc value

Three Detector Method

Jie Wen, Tsutomu Arakawa and John S. Philo

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

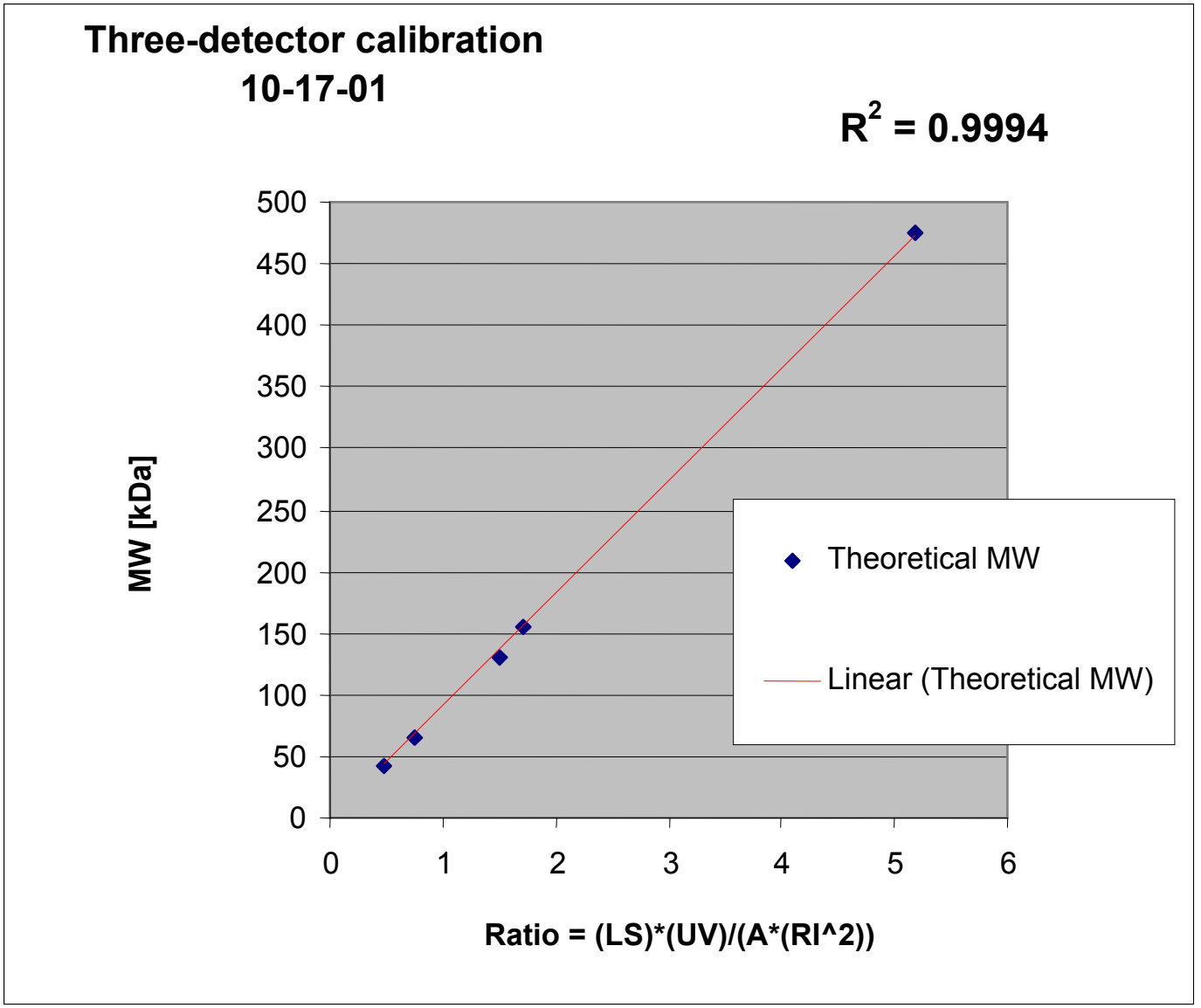
Yutaro Hayashi, Hideo Matsui and Toshio Takagi

Methods Enzymol 1989;172:514-28

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

MWp = 91.39 x [(LS)*(UV)/(A*(RI2))]

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

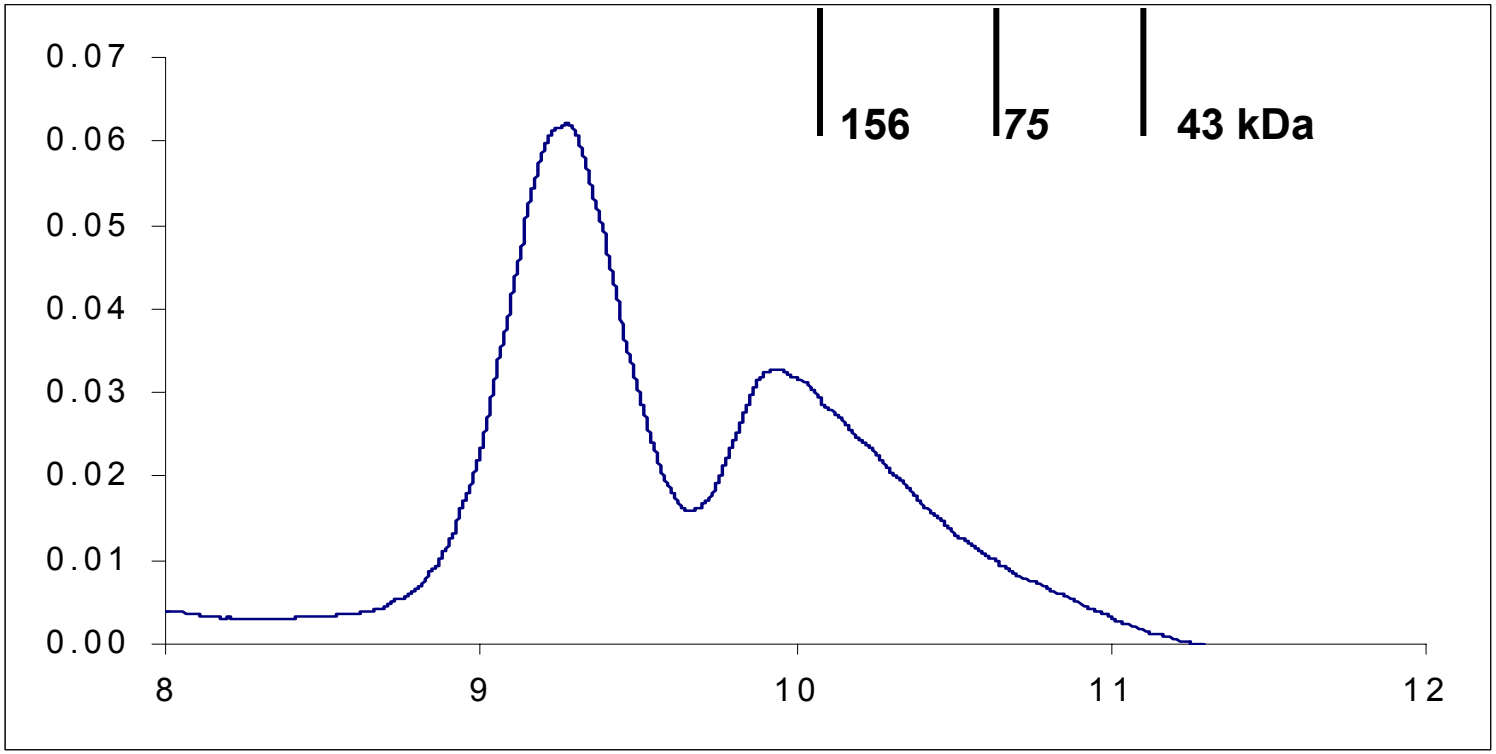


Determination of the oligomeric state of glycosylated protein

Example:

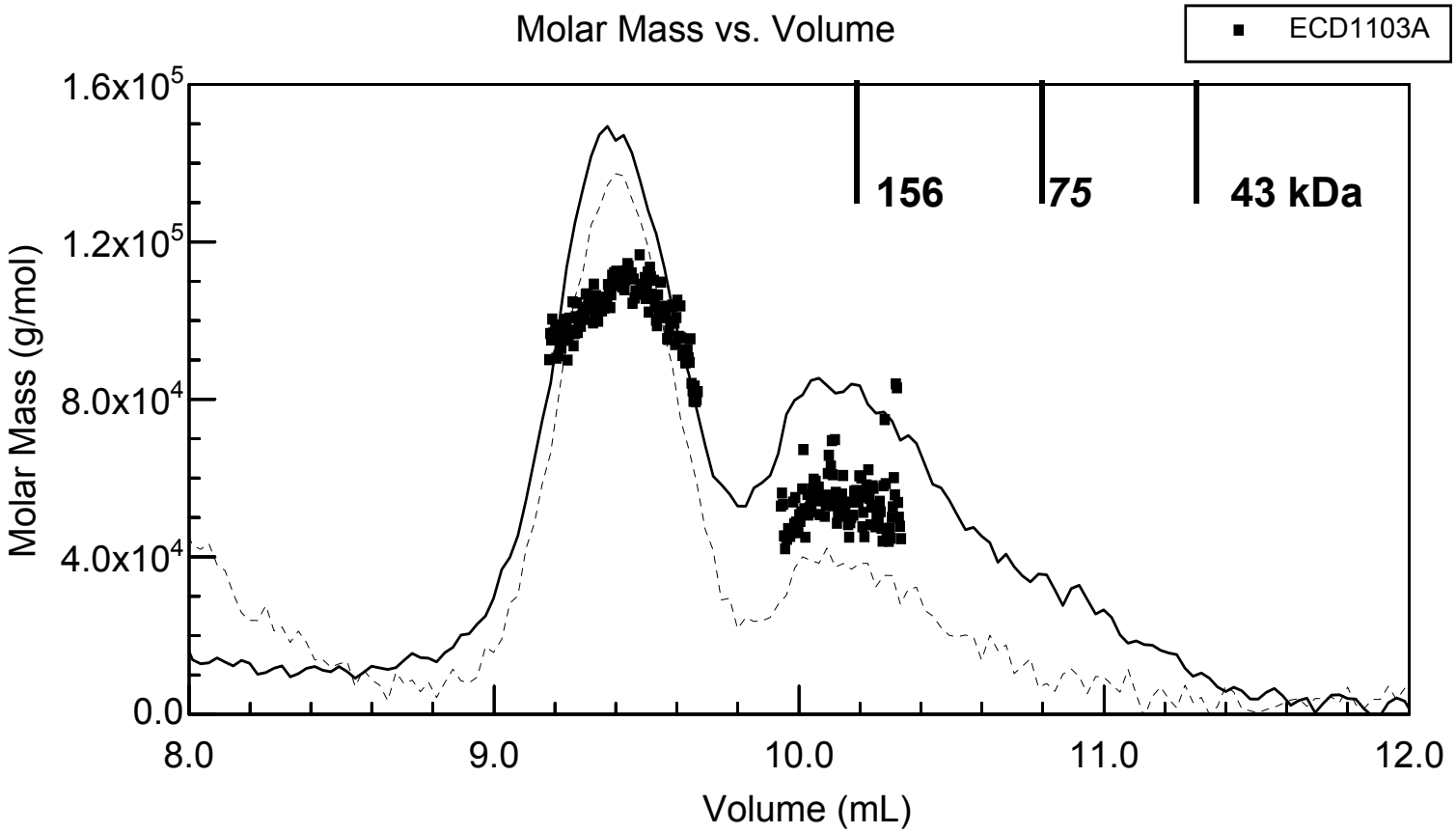
protein	*58 kDa*	*extracellular ANP-binding domain (ECD) of cell-surface receptor 16% of mass is sugar*
		dn/dc_t = 0.179 g/mL
	48 kDa	*polypeptide portion*
ligand	*2.7 kDa*	*atrial natriuretic peptide (ANP)*

Injected sample complex (ECD : ANP) 2:1



ECD $_{dimer}$ = 2 x 58 = 116 kDa (96 kDa) *ANP = 2.7 kDa*

Injected sample complex (ECD : ANP) 2:1



ECD $_{dimer}$ = 2 x 58 = 116 kDa (96 kDa) *ANP = 2.7 kDa*

ECD-ANP complex; ~3 μg

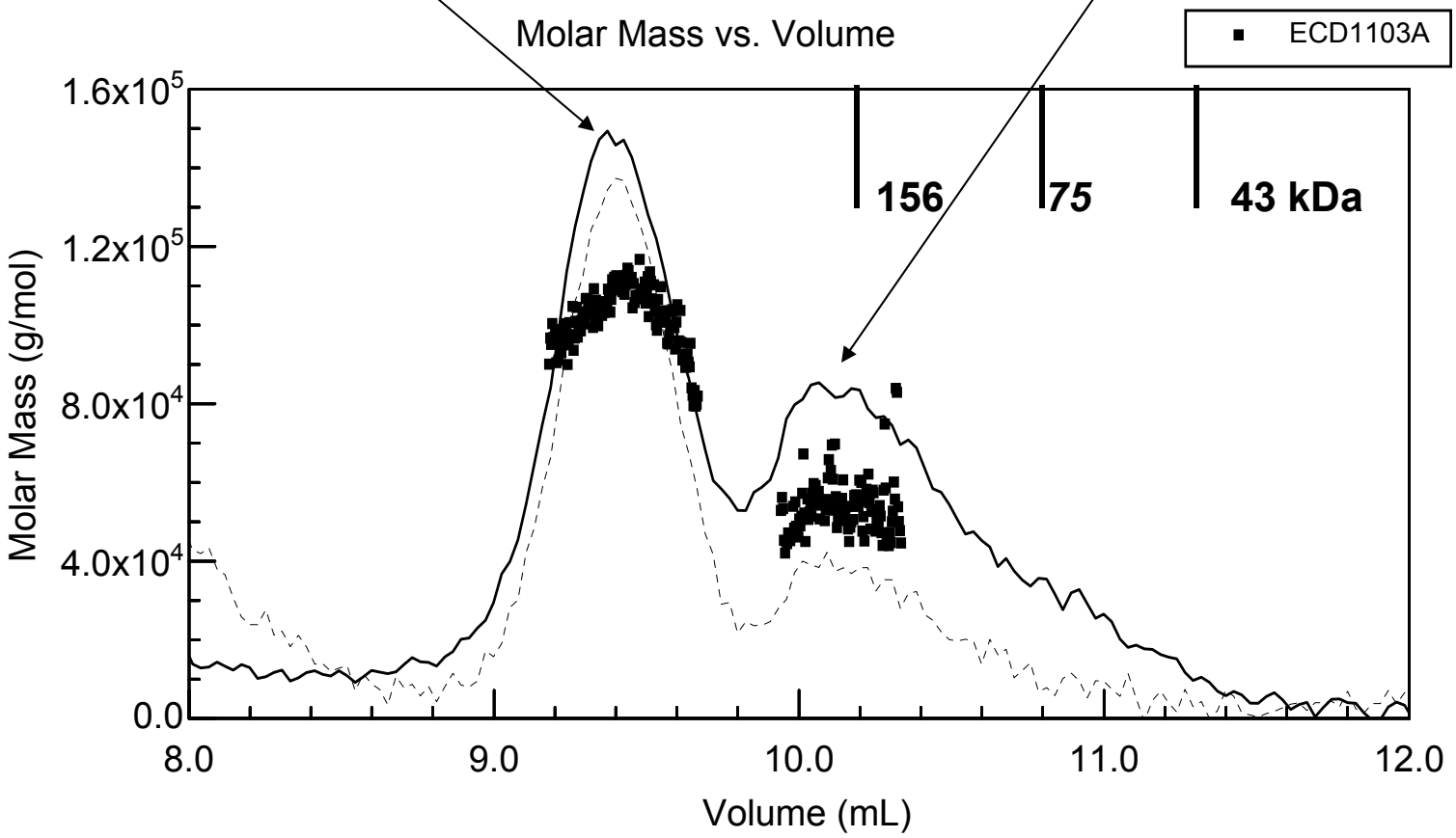
$MW_{glycoprotein}$ = 103 ± 10 kDa

$MW_{polypeptide}$ = 96 ± 7 kDa

ECD; ~2 μg

$MW_{glycoprotein}$ = 54 ± 6 kDa

$MW_{polypeptide}$ = 44 ± 5 kDa



ECD_{dimer} = 2 x 58 = 116 kDa (96 kDa)

ANP = 2.7 kDa

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)

Three Detector Method

Yutaro Hayashi, Hideo Matsui and Toshio Takagi

Methods Enzymol 1989;172:514-28

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

allows determination of MW polypeptide (oligomeric state of the protein)

allows determination of mass of detergent/lipids bound to polypeptide

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

δ *is mass of detergent and/or lipids per 1 gram of polypeptide*

Assumption : detergent does not produce any signal in UV

Determination of the oligomeric state of detergent solubilized protein

Example:

protein *47 kDa* *well characterized porin*

detergent

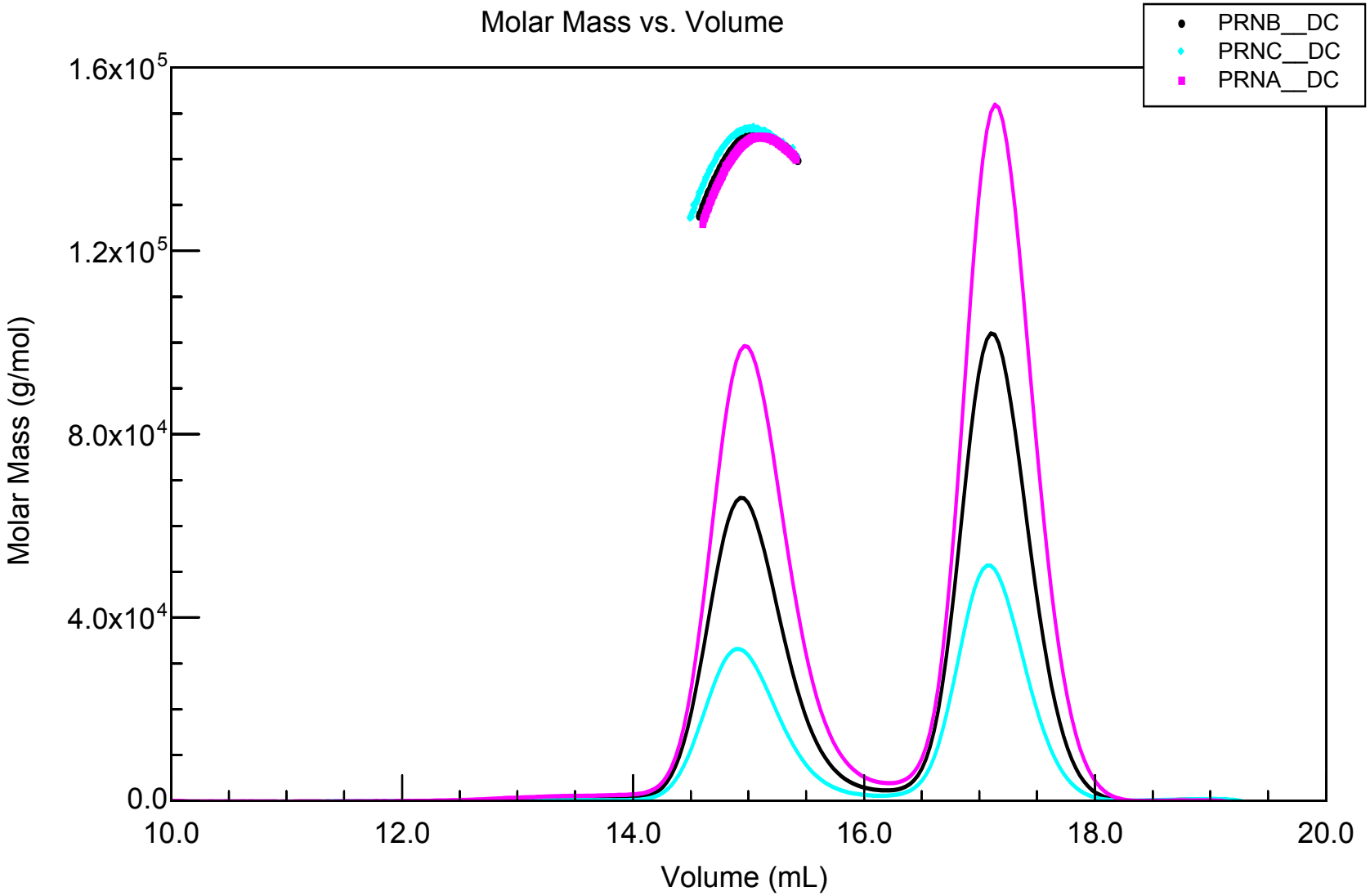
dodecyl maltoside (C12M) MW = 511 g/mol

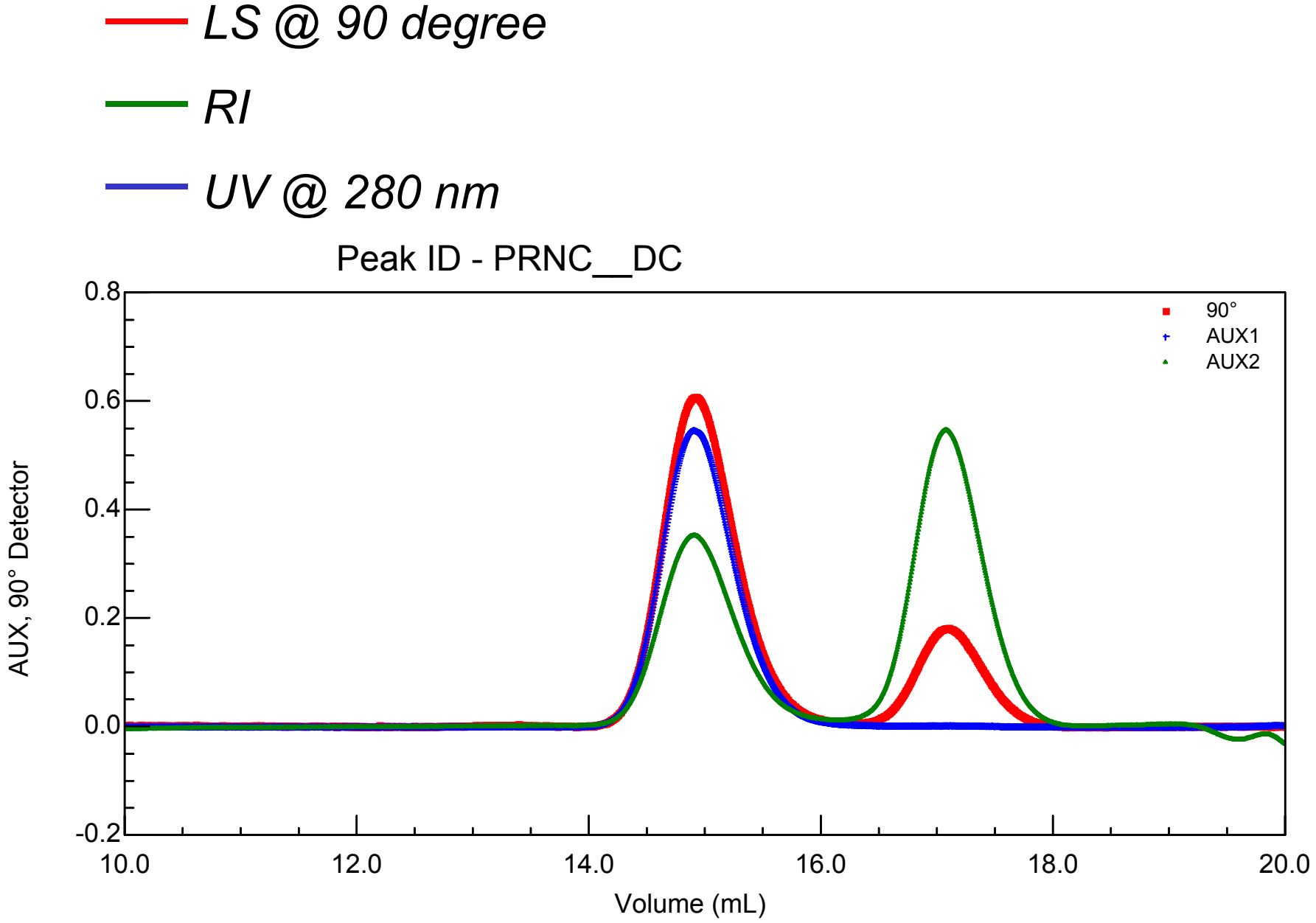
0.5g/L i.e. 0.05%

CMC = 0.008% micelle size 50-70 kDa

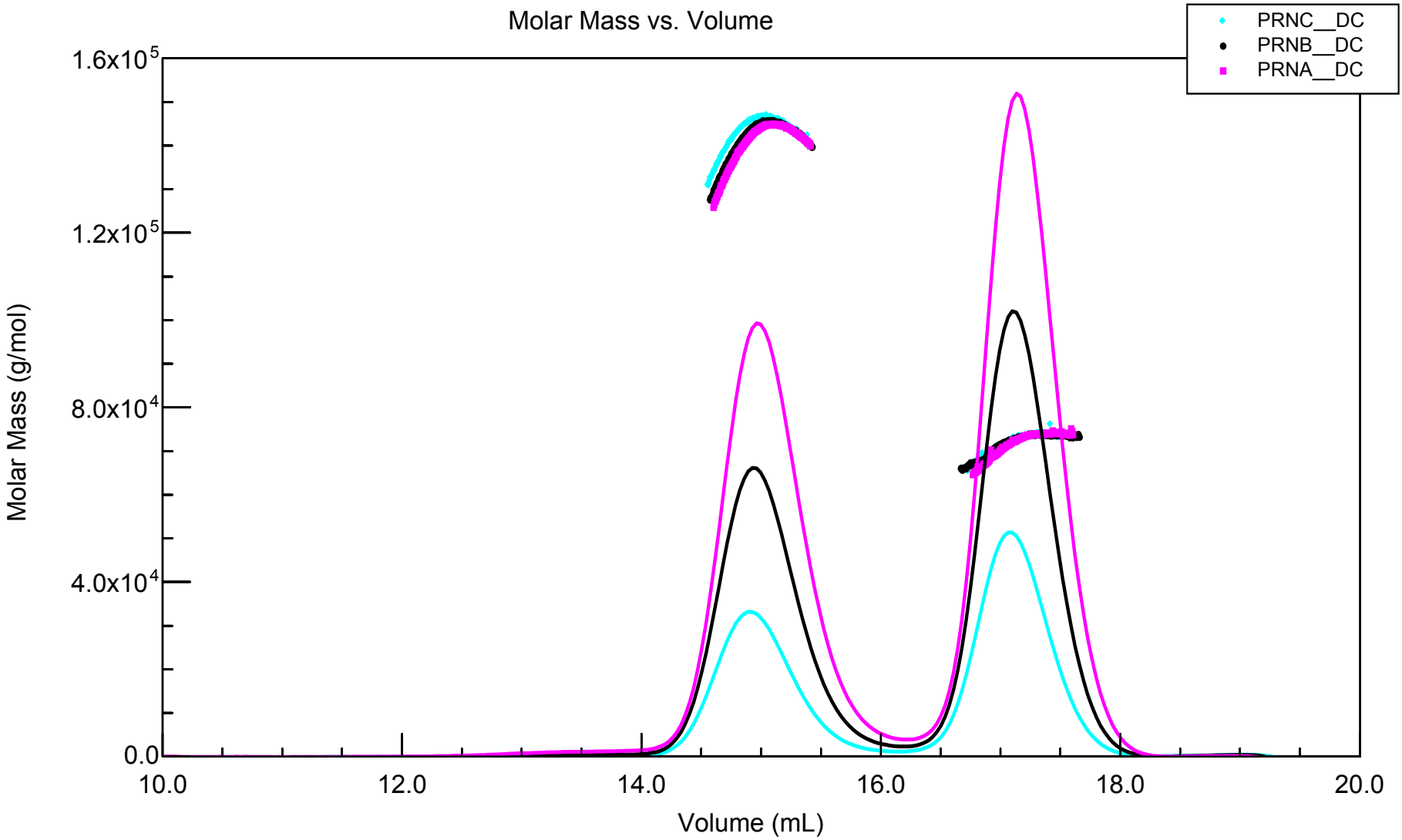
porin monomer = 47 kDa

MW = 149 ± 3 kDa trimer




LS @ 90 degree
RI
UV @ 280 nm
Peak ID - PRNC__DC
90°
AUX1
AUX2
AUX, 90° Detector
0.8
0.6
0.4
0.2
0.0
-0.2
10.0
12.0
14.0
16.0
18.0
20.0
Volume (mL)

C12M micelle size = 72 kDa



Determination of the oligomeric state of detergent solubilized protein

Example:

protein *33 kDa*

Detergent

dodecyl maltoside (C12M) MW = 511 g/mol

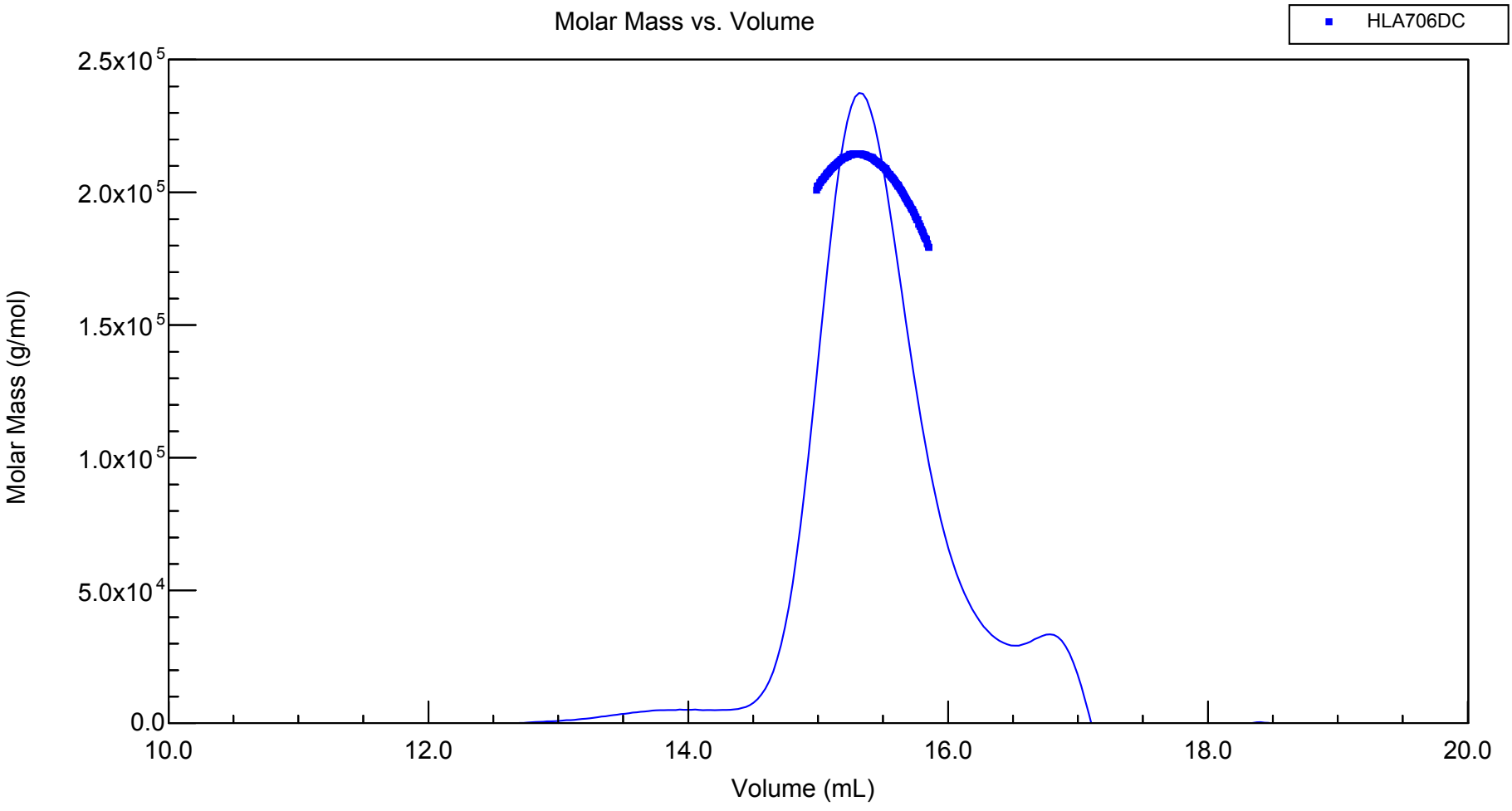
n-Dodecyl-β-D-Maltoside

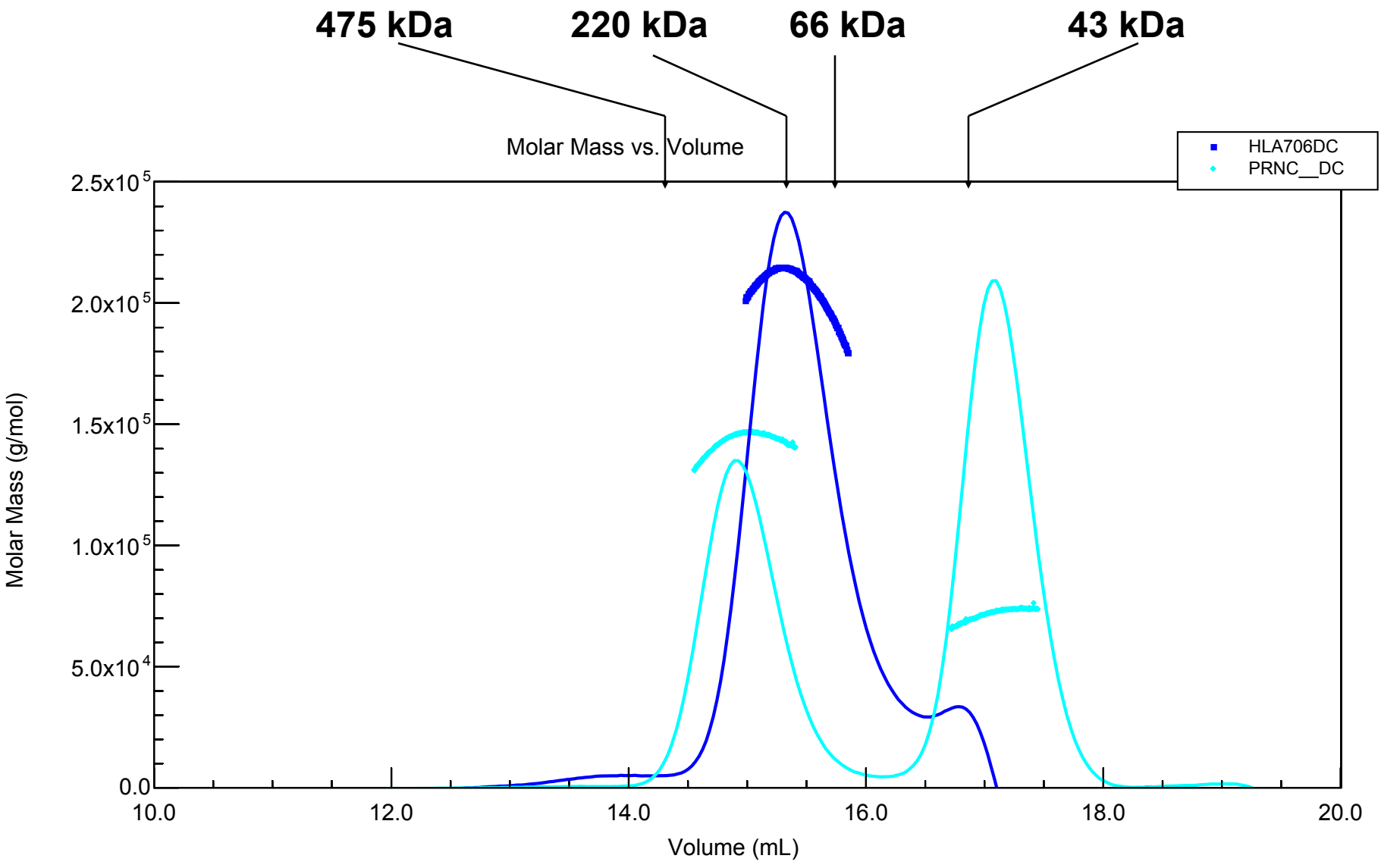
0.5g/L i.e. 0.05%

CMC = 0.008% micelle size 50-70 kDa

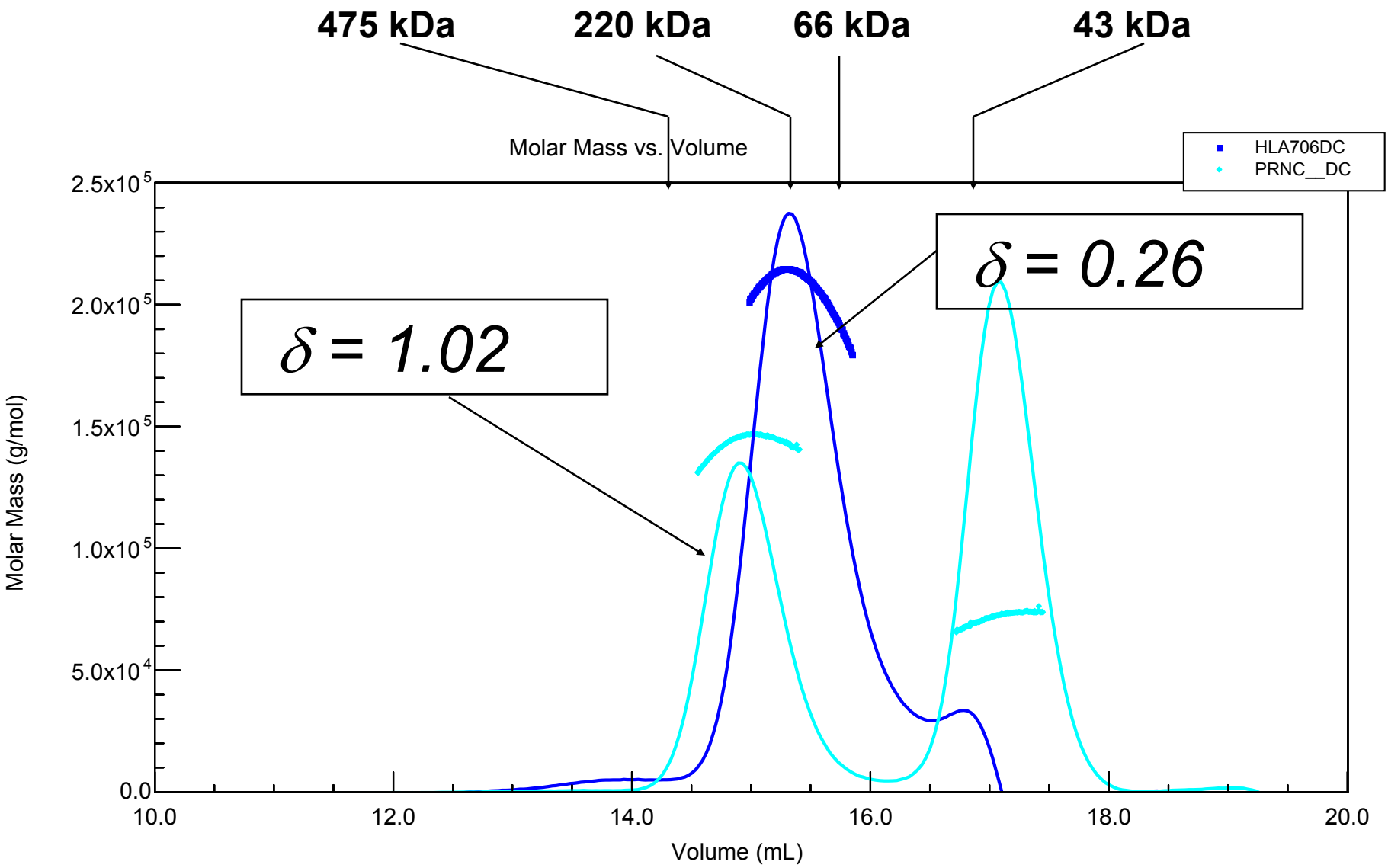
monomer = 33 kDa

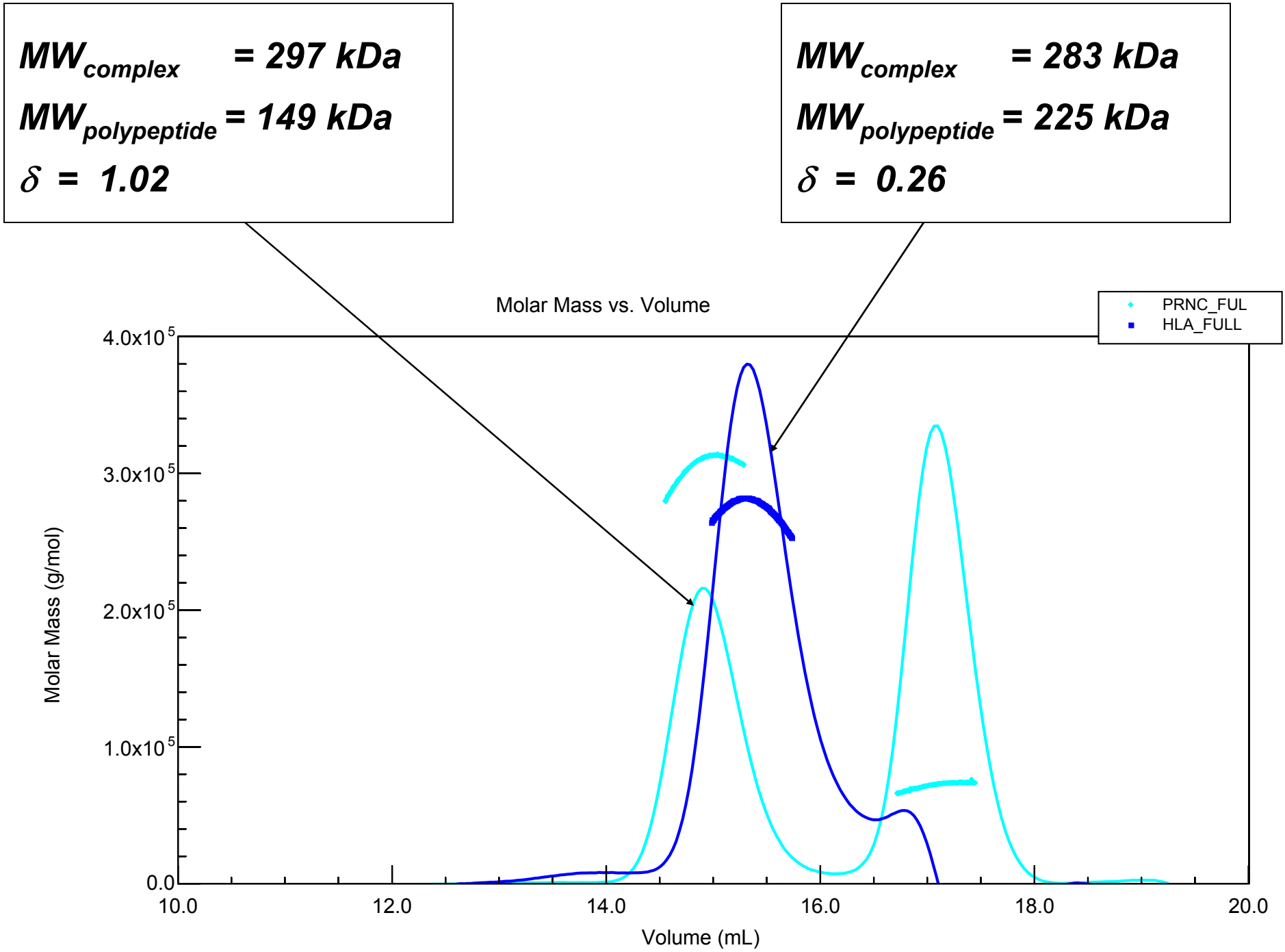
MW = 225 ± 13 kDa heptamer




475 kDa
220 kDa
66 kDa
43 kDa
Molar Mass vs. Volume
HLA706DC
PRNC__DC
Molar Mass (g/mol)
2.5x10^5
2.0x10^5
1.5x10^5
1.0x10^5
5.0x10^4
0.0
10.0
12.0
14.0
16.0
18.0
20.0
Volume (mL)

δ is mass of detergent and/or lipids per 1 gram of polypeptide




$MW_{complex}$ = 297 kDa
$MW_{polypeptide}$ = 149 kDa
δ = 1.02
$MW_{complex}$ = 283 kDa
$MW_{polypeptide}$ = 225 kDa
δ = 0.26
Molar Mass vs. Volume
PRNC_FUL
HLA_FULL
Molar Mass (g/mol)
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0.0
10.0
12.0
14.0
16.0
18.0
20.0
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 detergent solubilized membrane proteins

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

NIH

Users of SEC/LS Service

Wyatt Technology