

Advanced GFC Analysis for Proteins and Conjugated Proteins



Agenda

1. GFC (SEC) Measurements of Proteins with MALS

1.1 BSA

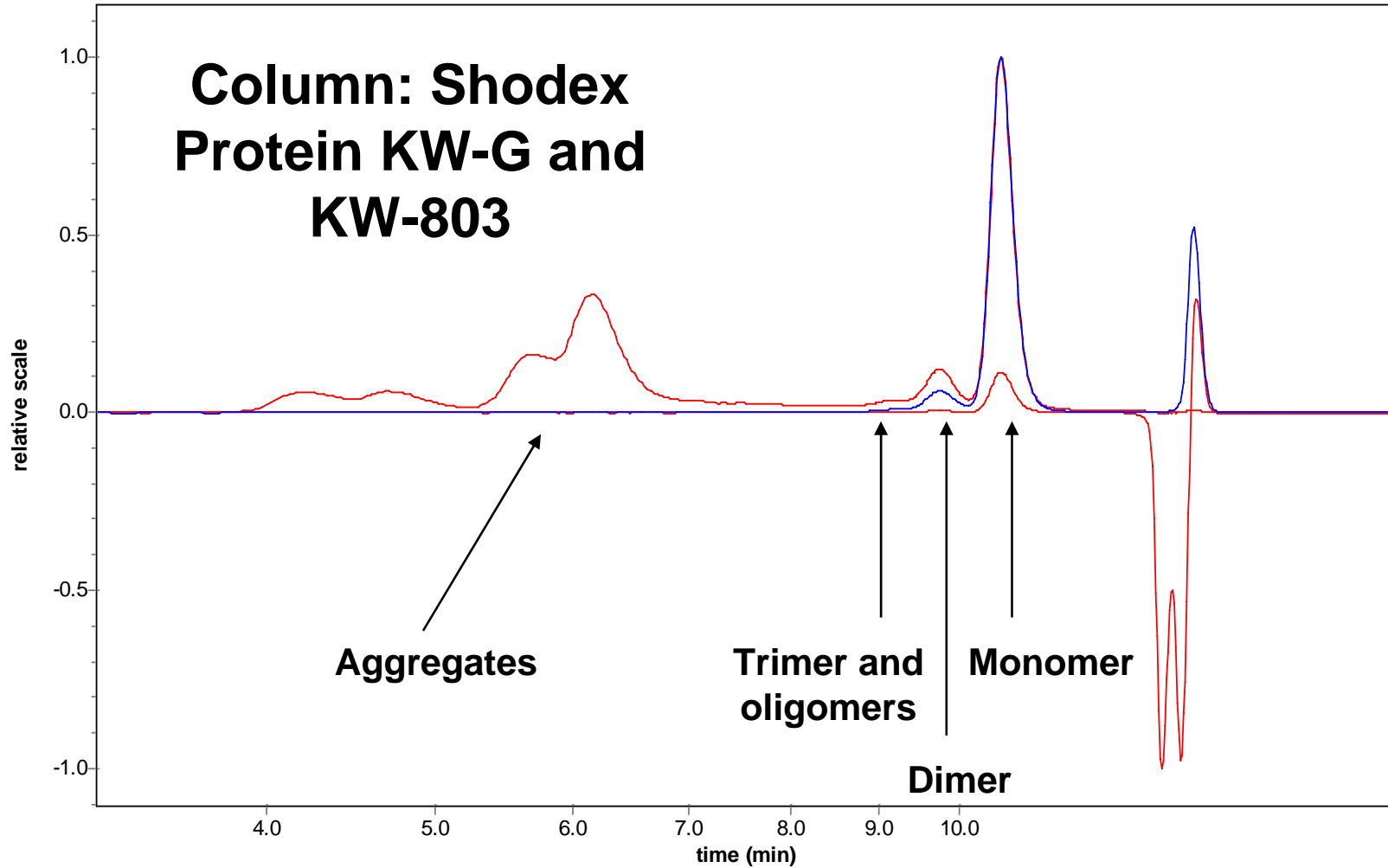
1.2 Thyroglobulin

2. GFC (SEC) Measurements of PEG-Proteins

1.1 GFC (SEC) Measurement of BSA Protein

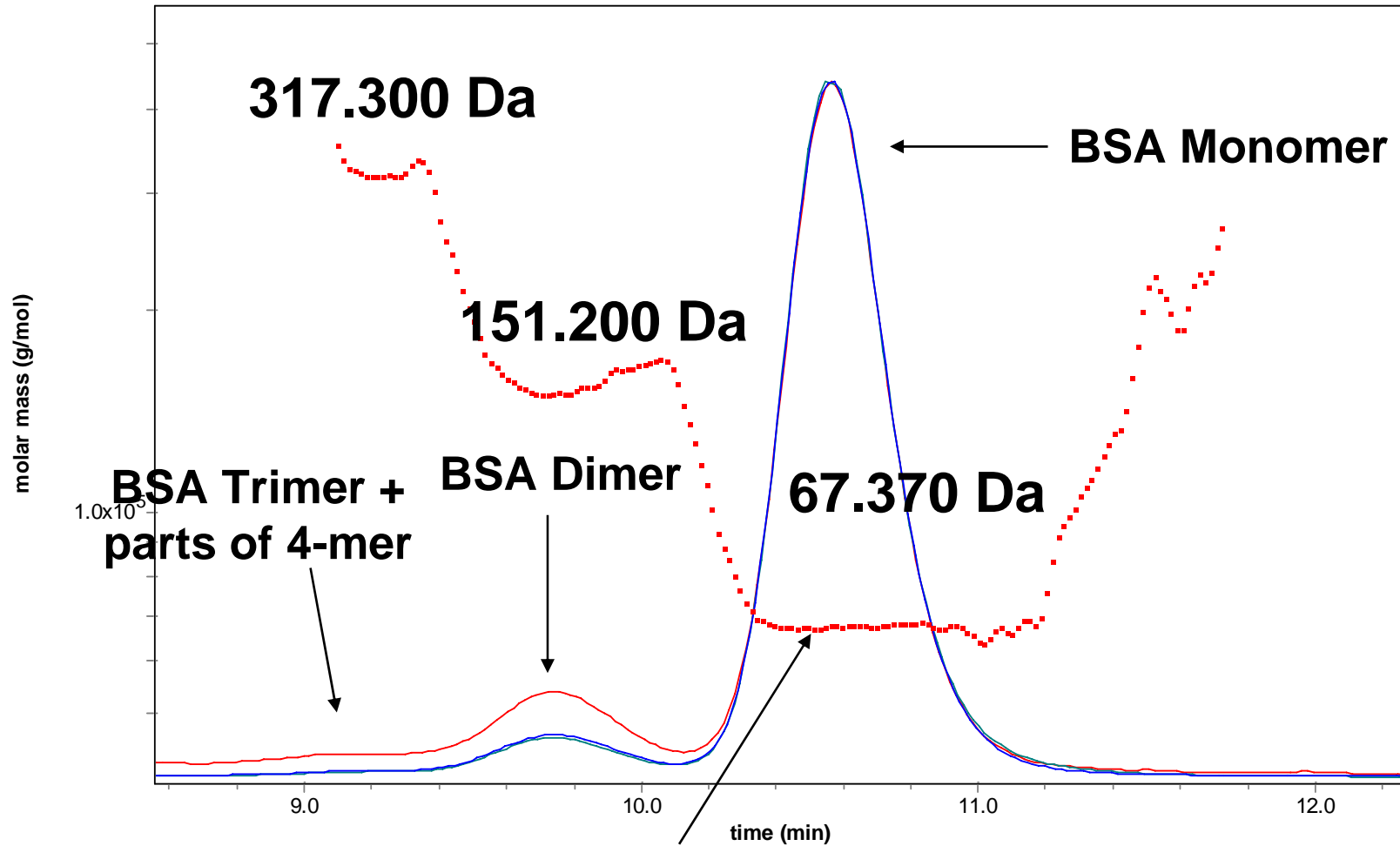
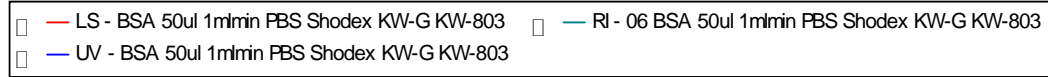
chromatograms

□ — LS - BSA 50ul 1ml/min PBS Shodex KW-G KW-803 □ — RI - BSA 50ul 1ml/min PBS Shodex KW-G KW-803 □ — UV - BSA 50ul 1ml/min PBS Shodex KW-G KW-803



1.1 GFC (SEC) Measurement of BSA Protein

molar mass vs. time



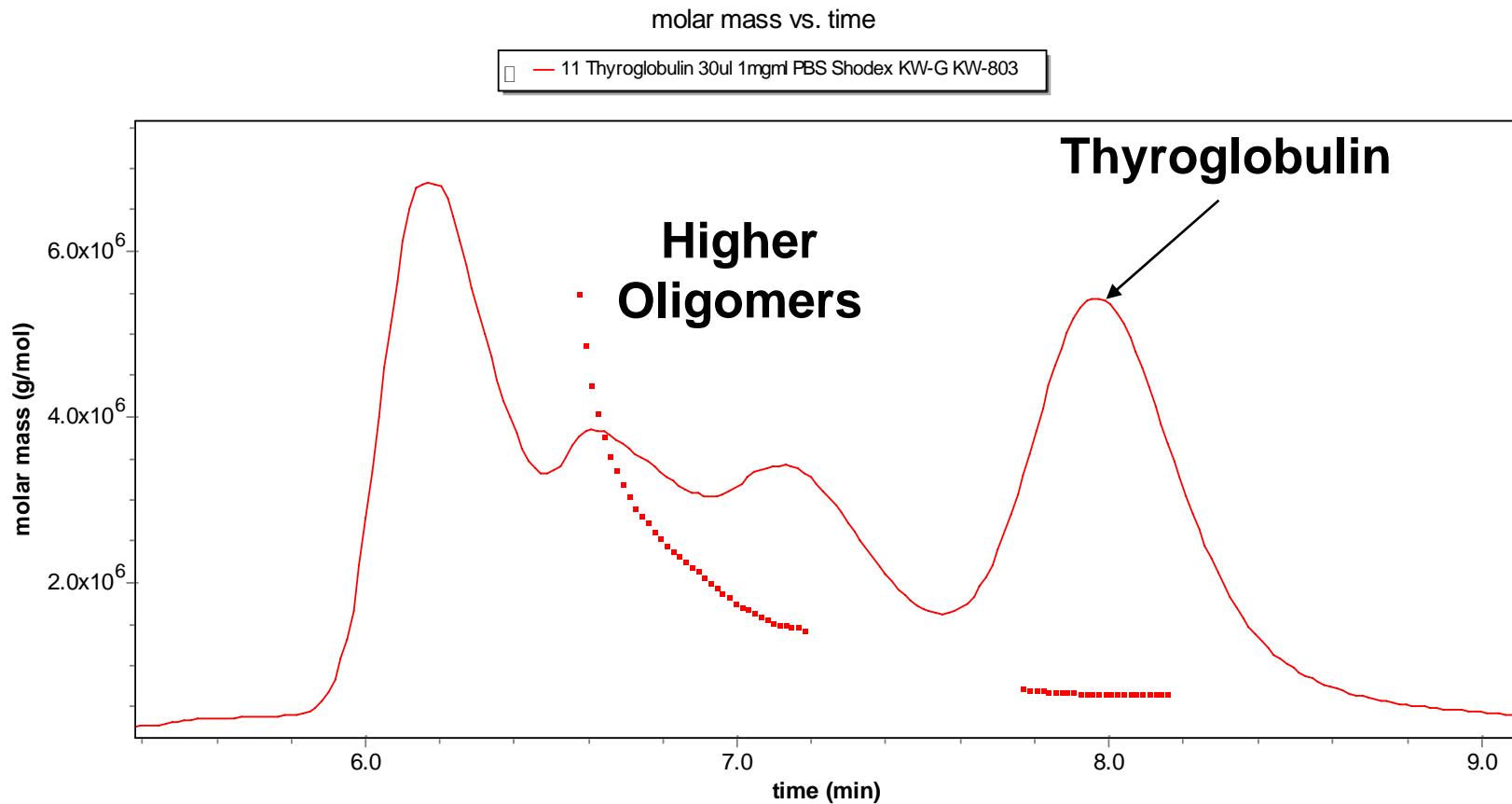
Absolute measured molar mass of BSA Monomer with MALLS; monodisper fraction

1.1 GFC (SEC) Measurement of BSA Protein

Possible results from a MALLS run with ideal columns specified for MALLS

- Absolute measured molar mass, no need for an molar mass standard
- identification of oligomers and aggregates
- Information about recovery and polydispersity
- Information dependent on separation ability of columns used → columns with low bleeding and high recovery (less adsorption) are necessary
Shodex Protein KW series are using Silica with a polymeric coating as base material → small pore size distribution and less adsorption

1.2 GFC (SEC) Measurement of Thyroglobulin



Measured molar mass Thyroglobulin: 668.300 Da;

Rz = 12.6 nm

Polydispersity in peak range: Mw/Mn = 1.00

Column: Shodex Protein KW-G + KW-803

Shodex Protein KW-800 Series

■ Standard Columns

Product Code	Product Name	Plate Number (TP/column)	Exclusion Limit		Particle Size (μm)	Maximum Pore Size (Å)	Column Size (mm) I.D.x L	Shipping Solvent
			(Pullulan)	(Protein)				
F6989000	PROTEIN KW-802.5	≥ 21,000	60,000	150,000	5	400	8.0 x 300	H ₂ O
F6989103	PROTEIN KW-803	≥ 21,000	170,000	700,000	5	1,000	8.0 x 300	H ₂ O
F6989104	PROTEIN KW-804	≥ 16,000	500,000	(1,000,000)	7	1,500	8.0 x 300	H ₂ O
F6700131	PROTEIN KW-G	(guard column)	—	—	7	—	6.0 x 50	H ₂ O

NEW

Shodex Protein KW-400 Series

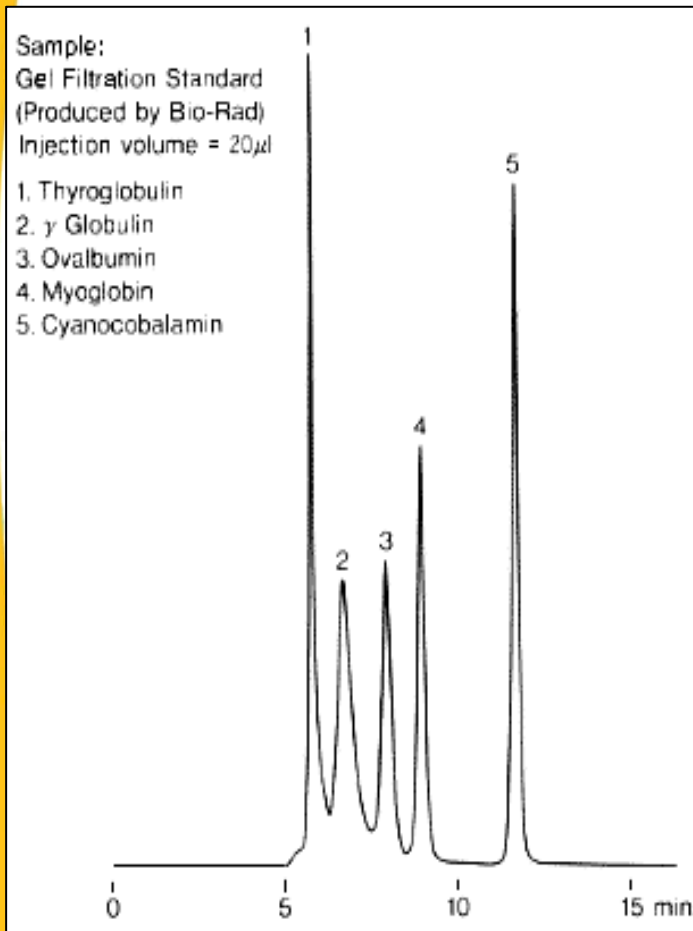
High performance and downsized column for protein analysis

■ High performance semi-micro columns

Product Code	Product Name	Plate Number (TP/column)	Exclusion Limit		Particle Size (μm)	Maximum Pore Size (\AA)	Column Size (mm) I.D.x L	Shipping Solvent
			(Pullulan)	(Protein)				
F6989201	NEW KW402.5-4F	$\geq 35,000$	60,000	150,000	3	400	4.6 x 300	H ₂ O
F6989202	NEW KW403-4F	$\geq 35,000$	150,000	600,000	3	800	4.6 x 300	H ₂ O
F6989203	NEW KW404-4F	$\geq 25,000$	500,000	(1,000,000)*	5	1,500	4.6 x 300	H ₂ O
F6989204	NEW KW405-4F	$\geq 25,000$	1,300,000	(20,000,000)*	5	2,000	4.6 x 300	H ₂ O
F6700132	NEW KW400G-4A	(guard column)	—	—	5	—	4.6 x 10	

1. High resolution compared with the current column
2. Lineup column with exclusion limit of 20,000,000

Standard Proteins and Recovery



Sample

- Gel Filtration Std:
1. Thyroglobulin
 2. gamma Globulin
 3. Ovalbumin
 4. Myoglobin
 5. Cyanocobalamin

Protein	Recovery(%)	
	KW-802.5	KW-803
γ Globulin	91	96
Bovine serum albumin	94	91
Ovalbumin	89	90
Myoglobin	92	95
Cytochrome c	100	98
Lysozyme	98	97
α.Chymotrypsinogen A	93	93

Column : **Shodex PROTEIN KW-802.5**
(8mmIDx300)

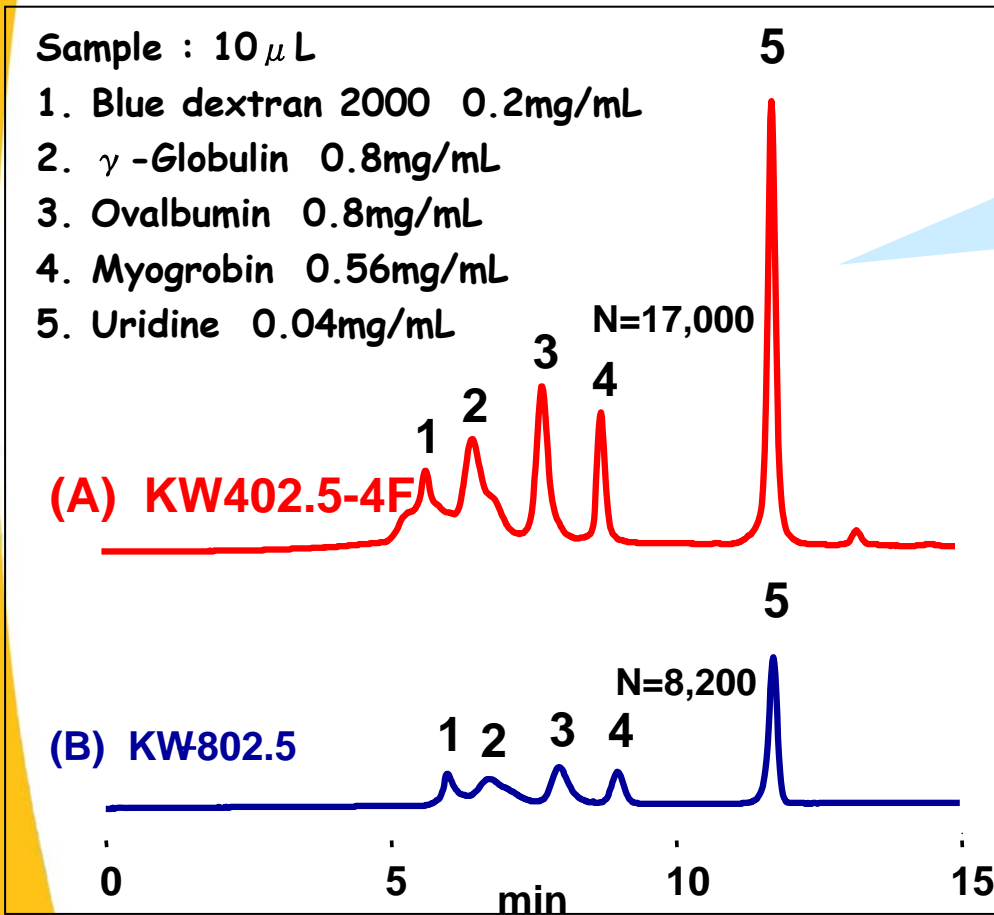
Eluent : 50mM Phosphate buffer
+ 0.3M NaCl(pH7.0)

Flow rate : 1.0mL/min

Detector : Shodex UV (280nm)

Column temp.: Ambient

Separation Performance KW-800 vs. KW-400



Comparison with existing columns

TPN... Twice higher
Sensitivity... 3 – 4 times higher

Columns : (A) Shodex KW402.5-4F
(4.6mmID x 300mm)
(B) Shodex PROTEIN KW-802.5
(8.0mmID x 300mm)

Eluent : 50mM Sodium phosphate buffer
+ 0.3M NaCl (pH7.0)

Flow rate : (A) 0.33mL/min,
(B) 1.0mL/min

Detector : UV (280nm) (semi-micro type cell)

Column temp. : 25 °C

10

Injector : Rheodyne 8125

2. GFC (SEC) Measurements of PEG-Proteins

$$M_w \propto \frac{I_{LS}}{\left(\frac{dn}{dc}\right)^2 \cdot c}$$

From DRI: $c_{DRI} = \frac{I_{DRI} \cdot DRI_{CC}}{\frac{dn}{dc}}$

DRI_{CC} : DRI calibration constant
 I_{DRI} : Intensity of the DRI signal

From UV: $c_{UV} = \frac{I_{UV} \cdot UV_{RF}}{\varepsilon \cdot L}$

UV_{RF} : UV response factor
 I_{UV} : Intensity of the UV signal

Basic equations of static light scattering

2. GFC (SEC) Measurements of PEG-Proteins

$$\left(\frac{dn}{dc}\right)_{\text{complex}} = \left(\frac{dn}{dc}\right)_{\text{protein}} \bullet X_{\text{protein}} + \left(\frac{dn}{dc}\right)_{\text{modifier}} \bullet (1 - X_{\text{protein}})$$

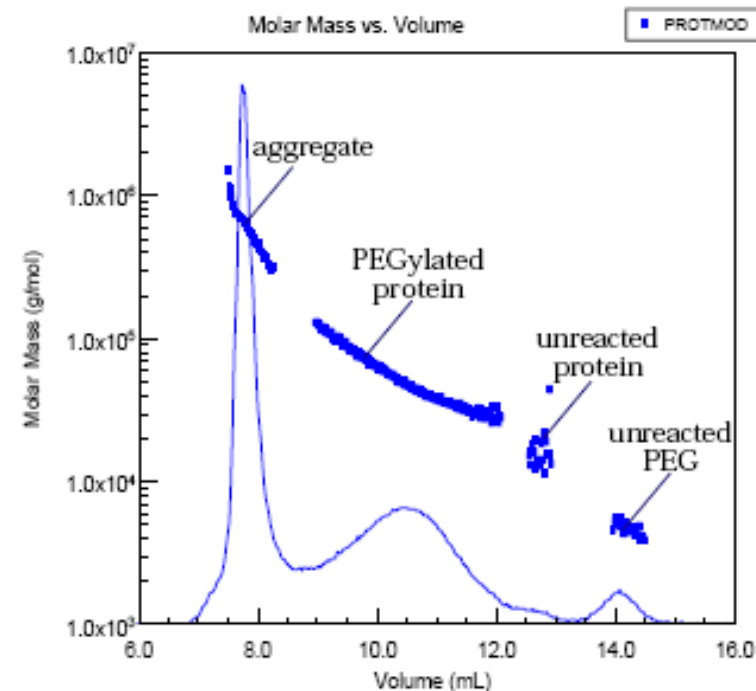
$$\epsilon_{\text{complex}} = \epsilon_{\text{protein}} \bullet X_{\text{protein}} + \epsilon_{\text{modifier}} \bullet (1 - X_{\text{protein}})$$

**Equations for modified
Proteins**

Light Scattering with Conjugated Proteins

What can be done with a combination of SEC and LS?

1. Protein-PEG-Protein bonding (confirmation that the PEG was successfully bonded)
2. Degree of PEGylation
3. Aggregation detection
4. Extent of reaction



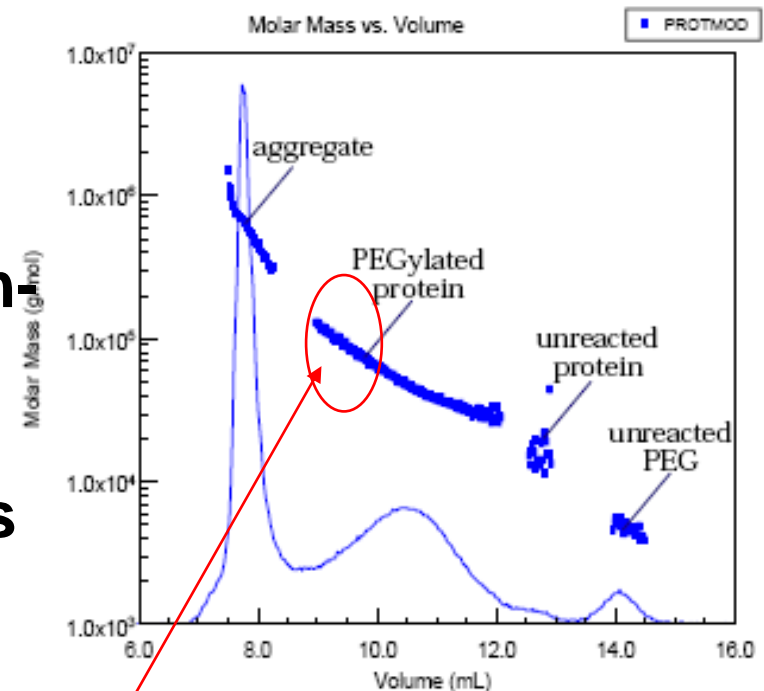
Caution pay attention to the changing dn/dc value (0.135 for pure PEG and 0.185 for pure protein)

UV – LS – RI detection is necessary

Light Scattering with Conjugated Proteins

1. Protein-PEG-Protein bonding

→ Possibility to detect Protein-PEG-Protein complexes which could affect the pharmacological properties of the sample

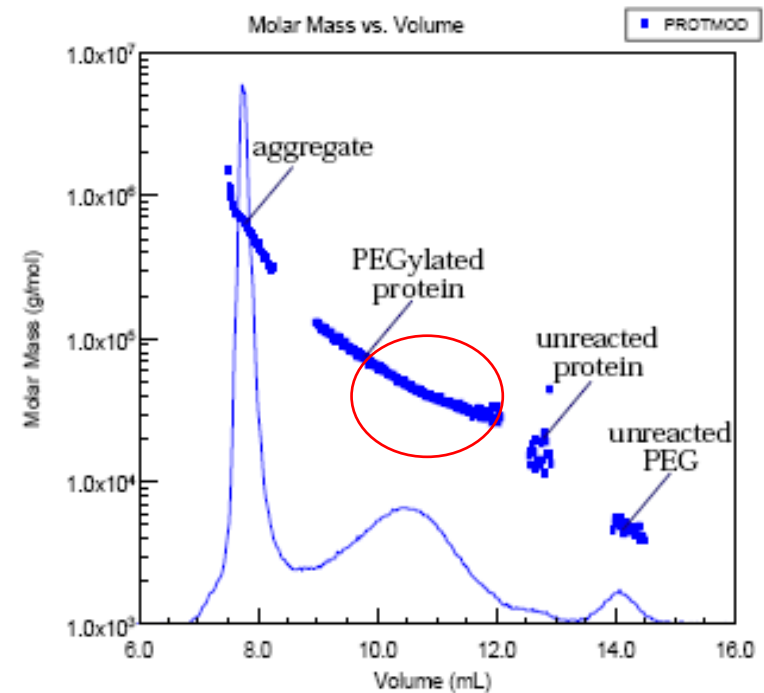


Protein-PEG-Protein complex with around 100 KDa; max PEGylation with this specific protein just 56 KDa

Light Scattering with Conjugated Proteins

2. Degree of PEGylation

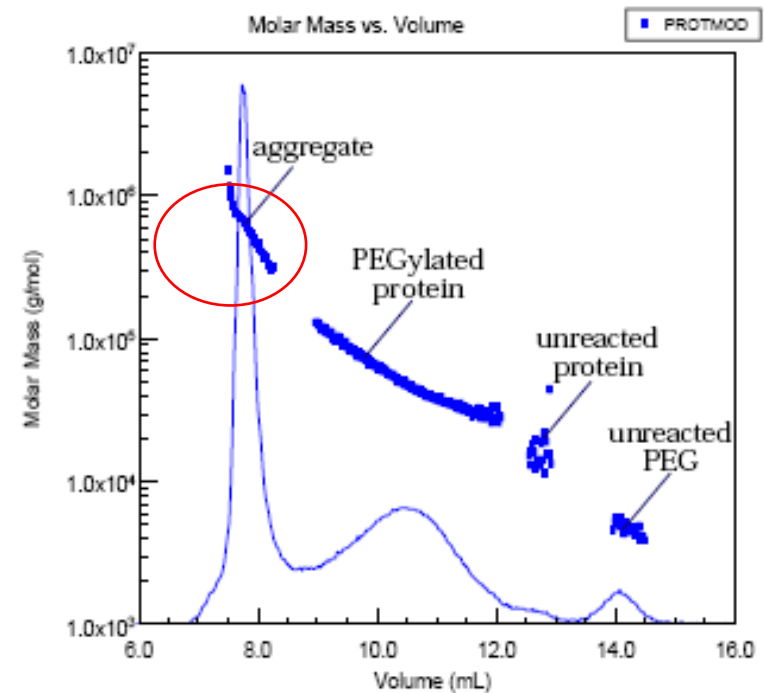
→ **Determination of the PEGylation degree is possible with LS, because Molar Mass is measured absolutely and no confirmation must be assumed**



Light Scattering with Conjugated Proteins

3. Detection of Aggregates

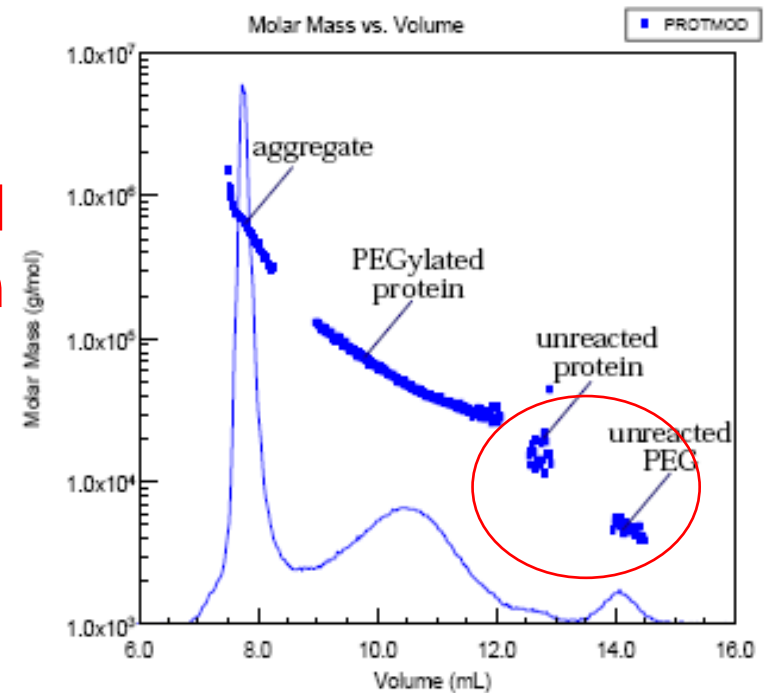
→ **Determination of Aggregates is possible**



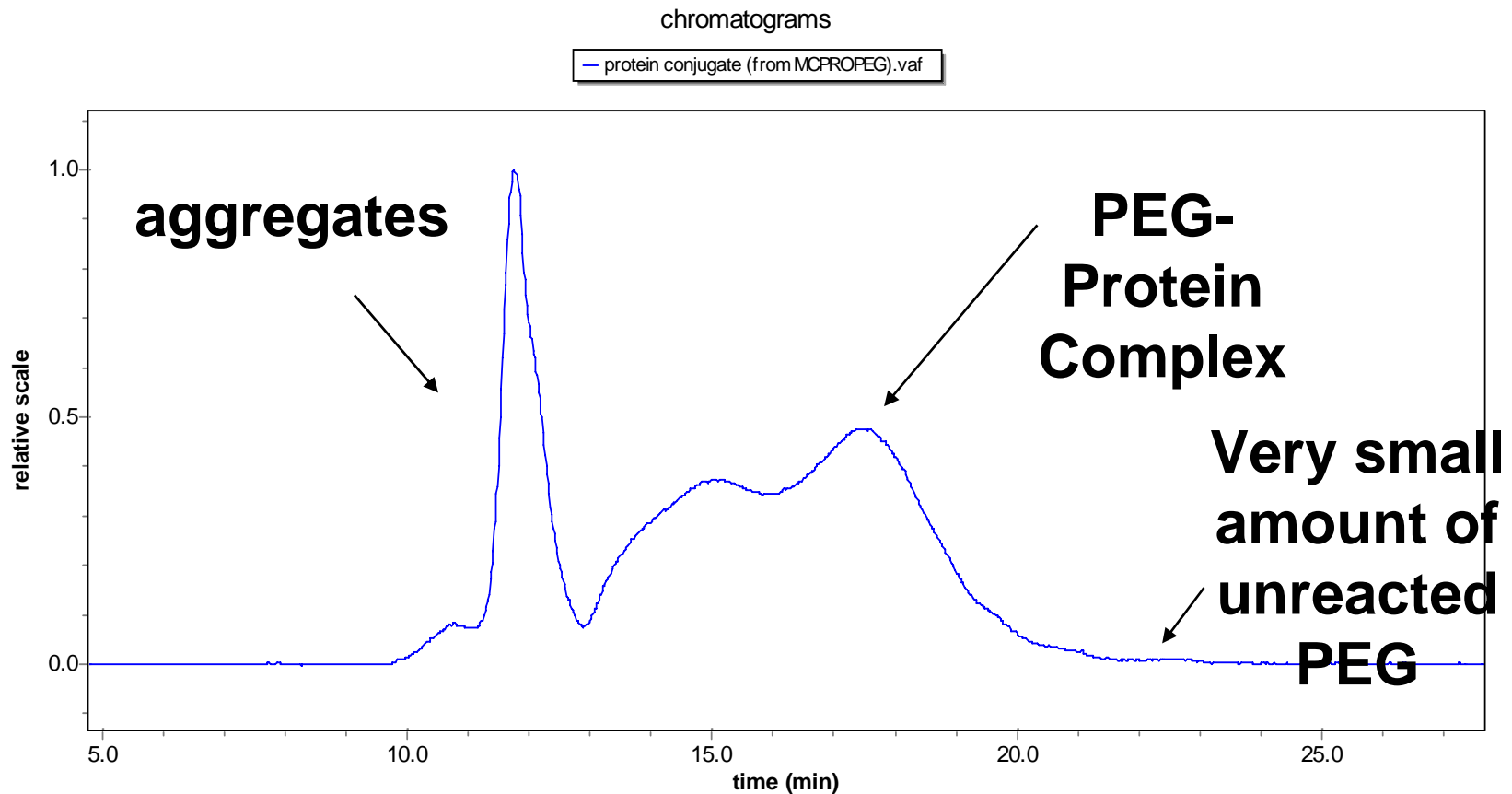
Light Scattering with Conjugated Proteins

4. Extend of Reaction

→ **Determination of unreacted PEG and unreacted Protein is possible**



2. GFC (SEC) Measurements of PEG-Proteins



dn/dc (pure protein) = 0.185

$x_{\text{protein}} = 0,631$
(calculated)

dn/dc (pure PEG) = 0.135

dn/dc (PEG-Protein) = 0.167 (calculated)

2. GFC (SEC) Measurements of PEG-Proteins

Mw (PEG-Protein): 100.500 Da

Mn (PEG-Protein): 83.130 Da

Mw (pure Protein): 60.111 Da

Mn (pure Protein): 53.338 Da

Mw (pure PEG): 40.360 Da

Mn (pure PEG): 27.010 Da

→ Degree of pegylation: 36.9 %

→ one PEG 40000 is bonded to one monomeric Protein

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Technology Corporation in Santa Barbara (USA)
for her help in doing some measurements**

**Measurements are done on Wyatt Technologies
MALS instruments**