Triple Detector Size Exclusion Chromatography for biomolecules characterization

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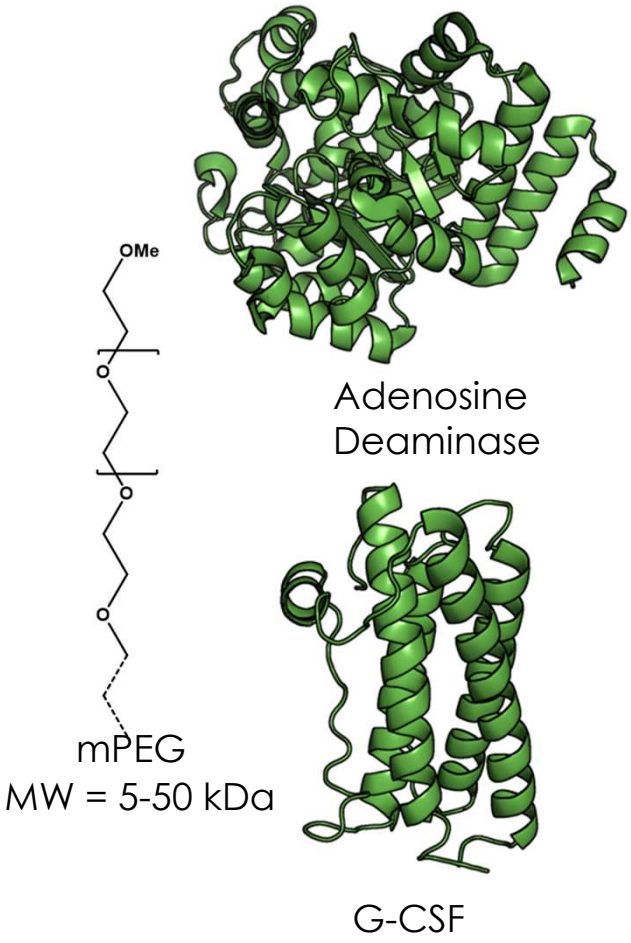
Agenda

- Application Introduction
- SEC Principle
- Multi detection
- Case study : Protein PEGylation

Application Introduction

Application introduction

- PEGylation can give a number of advantages:
  - Improved therapeutic half-life
    - Increased efficacy
    - Reduced frequency and level of dosage required for effective treatment
  - Reduced immunogenicity
  - Improved solubility and stability



SEC Principle

SEC Principle

Sample mixture
- Dissolved in the mobile phase

Separation

Stationary phase

Mobile phase
- Separates the analyte to be measured from other molecules in the mixture and allows it to be isolated.

Detectors

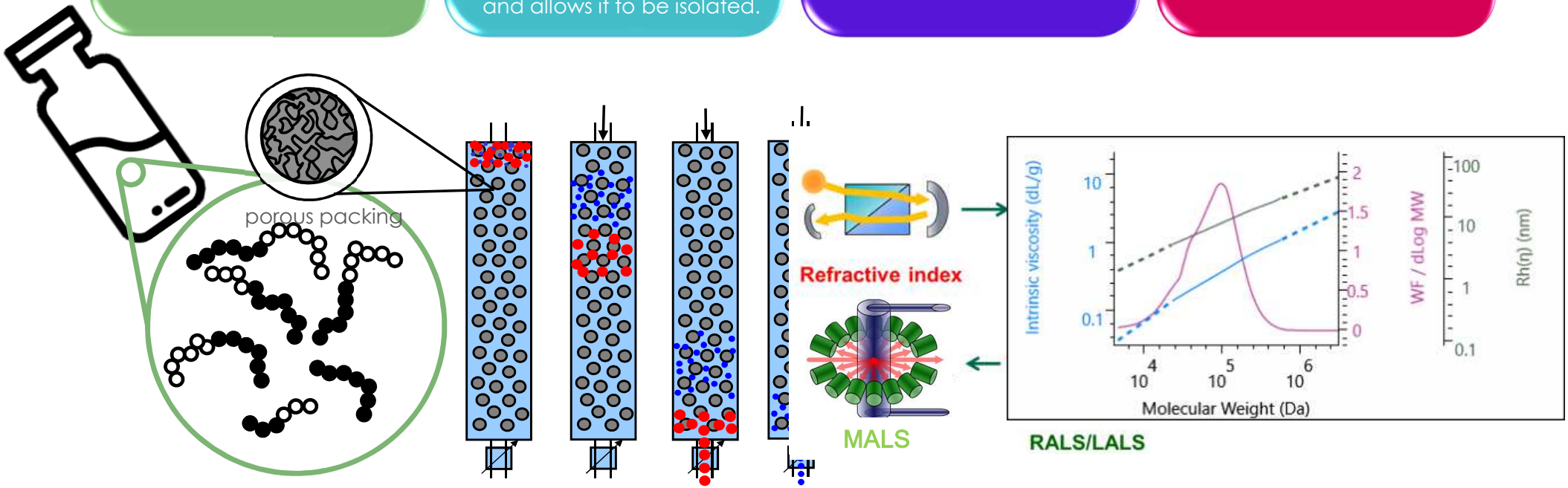
Results

Molecular weight

Size

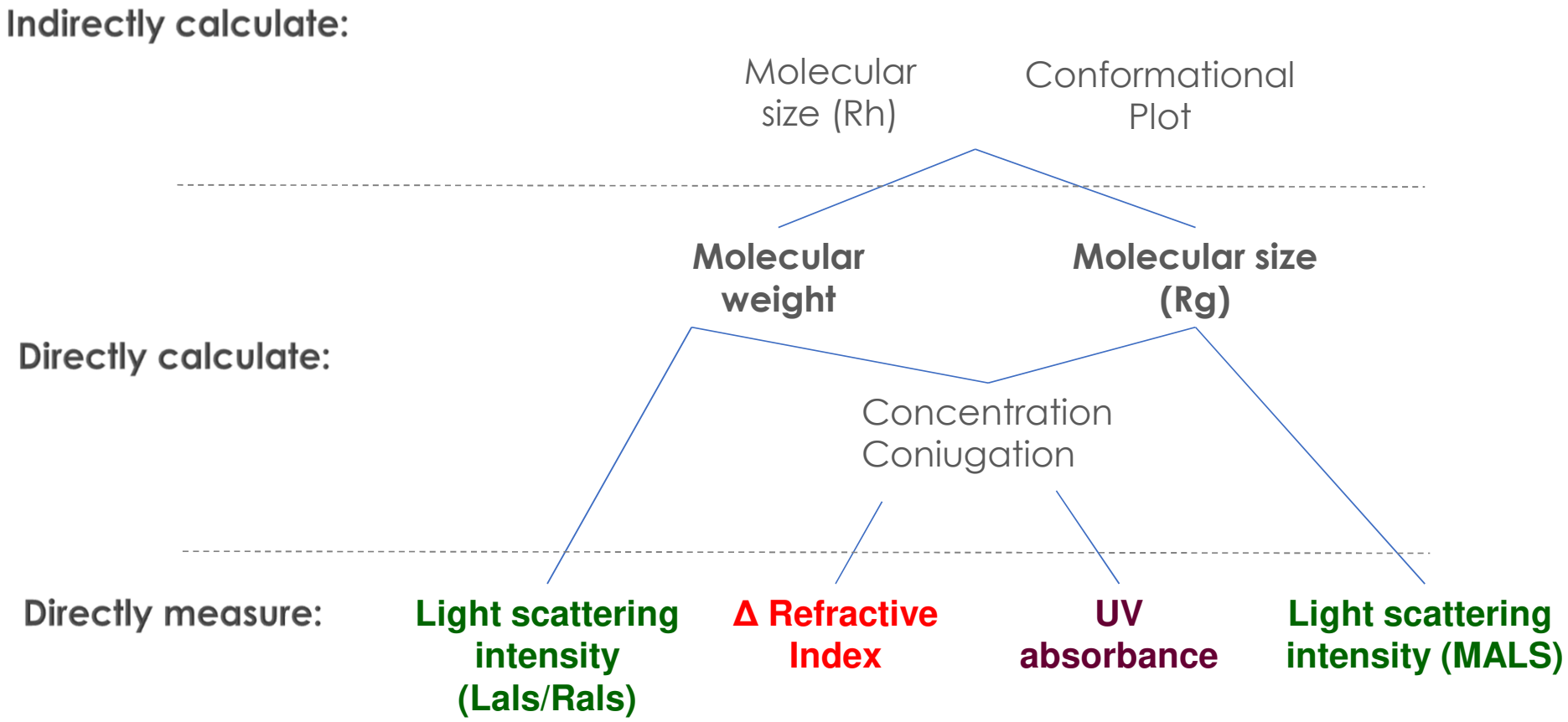
Amount

Conyugation



Introduction

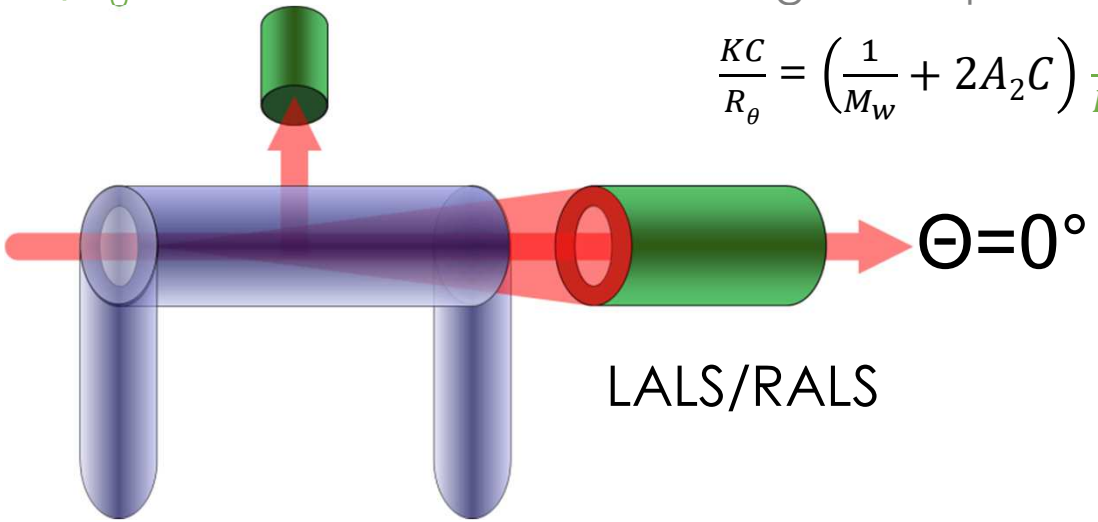
Advanced SEC

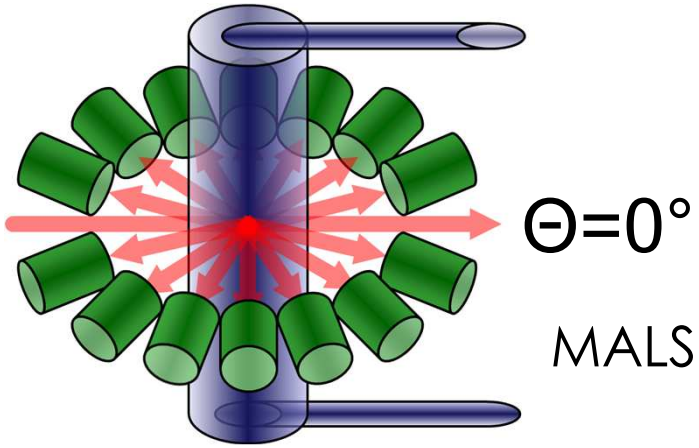


Light Scattering

- Light scattering is used to measure ***absolute* molecular weight** of samples independent of column retention volume
- The LS detectors are the RALS/LALS (Right Angle Light Scattering / Low Angle Light Scattering) detector and or MALS (Multi Angle light Scattering)
- $1/P_\theta$ is a term that defines the angular dependence of the scattered light

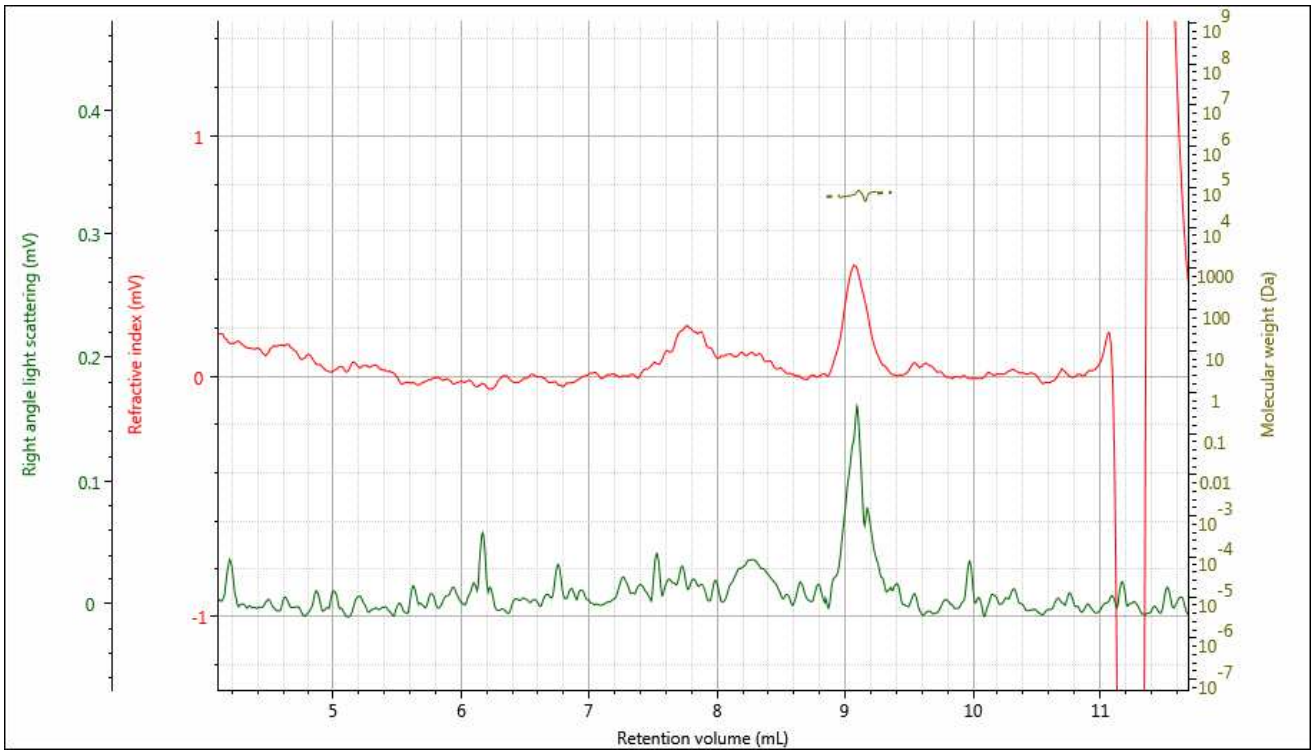
$$\frac{KC}{R_\theta} = \left(\frac{1}{M_w} + 2A_2C\right)\frac{1}{P_\theta}$$





OMNISEC REVEAL LALS/RALS sensitivity

- Molecular weight quantification at 100 ng!
- BSA 66 KDa in PBS 0.05 mg/ml, 2 µL injection
- Accurate calculation of molecular weight



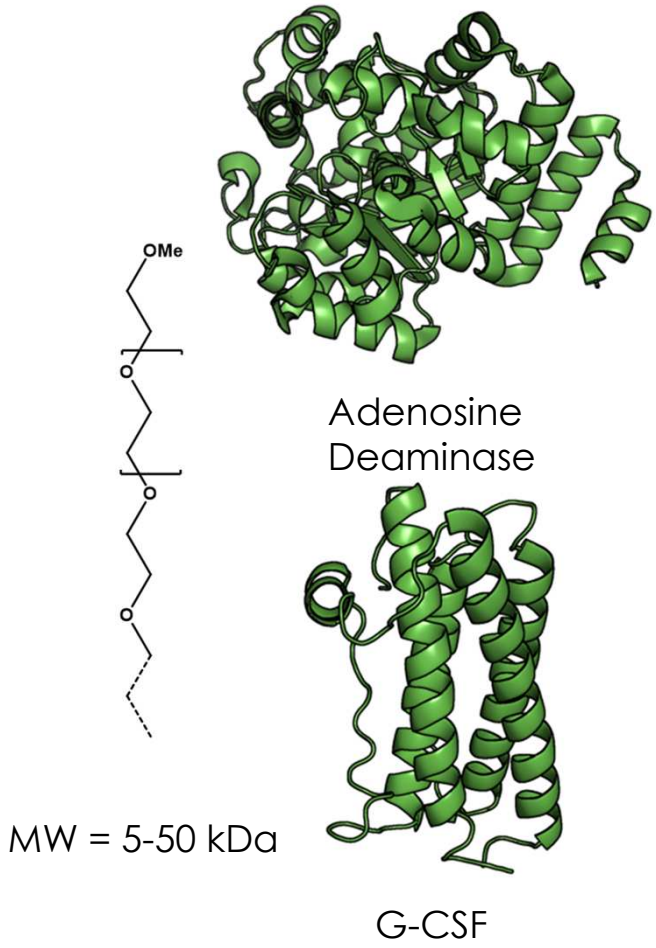
RV (mL)	9.083
Mn (g/mol)	63,800
Mw (g/mol)	65,070
Mz (g/mol)	66,290
Mw/Mn	1.02

Case Study

Case study: Protein PEGylation

- PEGylated Proteins

- PEGylation: covalent attachment of poly(ethylene glycol) (PEG) to a protein surface
- PEG: Highly water-soluble polymer with low immunogenicity and FDA-approved
- Well-established, safe and successful technology
- PEGylation can give a number of advantages:
  - Improved therapeutic half-life
    - Increased efficacy
    - Reduced frequency and level of dosage required for effective treatment
  - Reduced immunogenicity
  - Improved solubility and stability

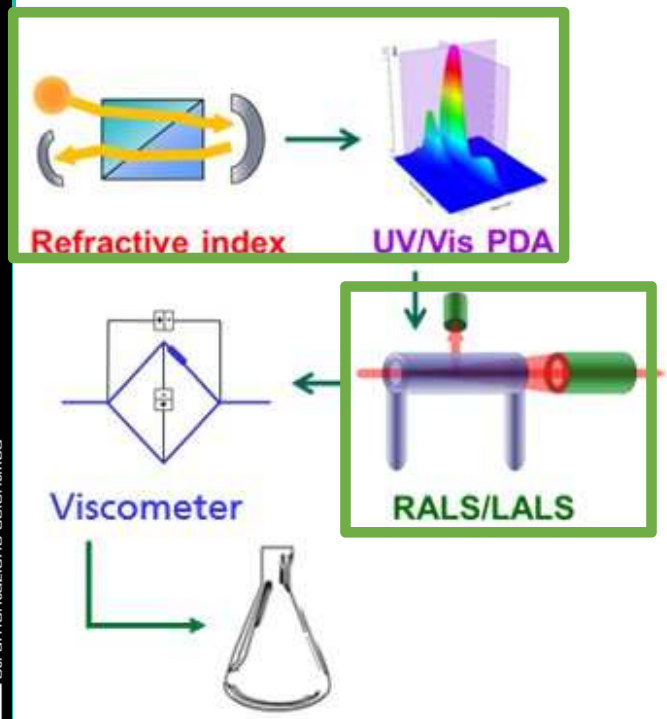


Aim of the experiment

- To carry out PEGylation on a protein
- To analyse the crude PEGylated protein using the SEC system OMNISEC from Malvern Panalytical
  - Understand the sample constituents at the molecular level

Why compositional analysis?

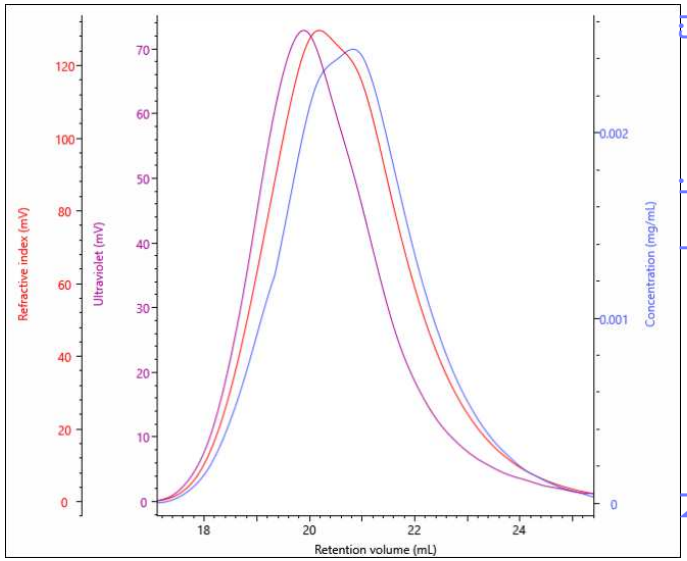
- The OMNISEC SEC system
  - Two different species affect the detector signals in different ways, complicating the calculations
  - Combination of RI and UV/Vis detectors allow for:



Concentration — dn/dc and dA/dc of both components

Absolute molecular weight

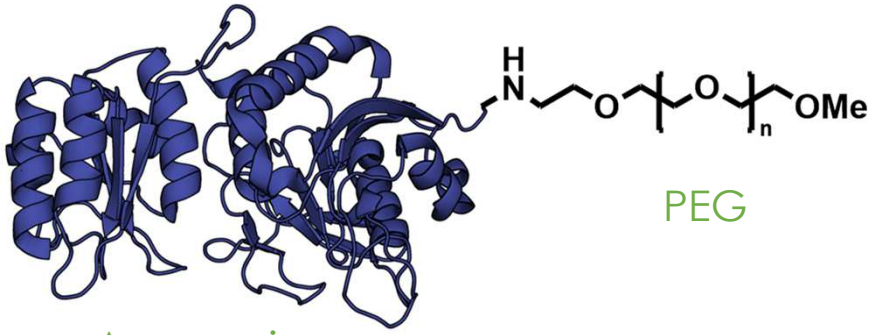
Composition — Components' weight fractions



New concentration profile

OMNISEC compositional analysis

- PEGylated proteins



Asparaginase

PEG

$$RI\ output \propto C_{Asp} \cdot \left(\frac{dn}{dc}\right)_{Asp} + C_{PEG} \cdot \left(\frac{dn}{dc}\right)_{PEG}$$

$$UV\ output \propto C_{Asp} \cdot \left(\frac{dA}{dc}\right)_{Asp}$$

- RI – Asparaginase and PEG
- UV @220 nm - Asparaginase

- Concentrations of PEG and Asparaginase
- Mw of the complex
- Mw of PEG and Asparaginase
- Weight fractions of PEG and Asparaginase
- Conjugation degree (PEG/Asparaginase ratio)

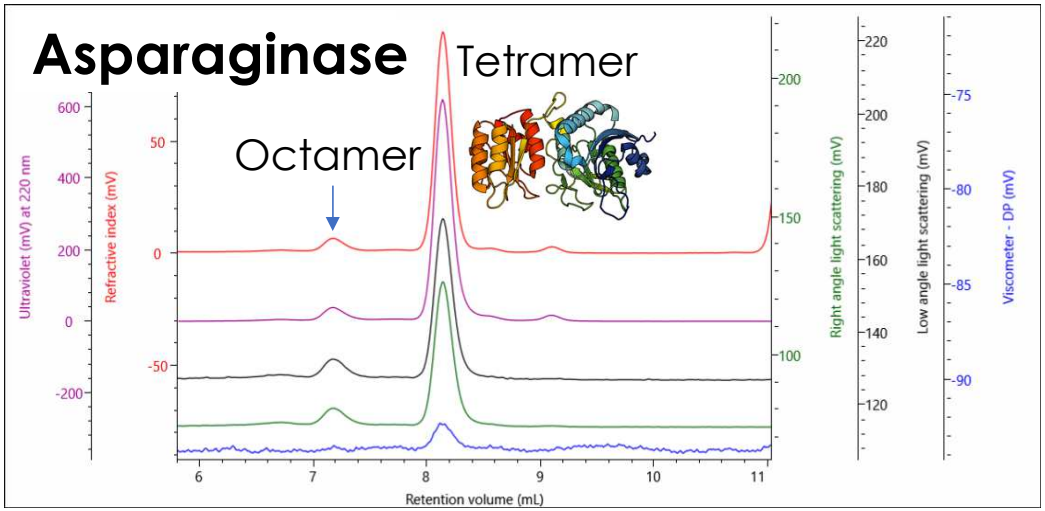
Chromatography conditions

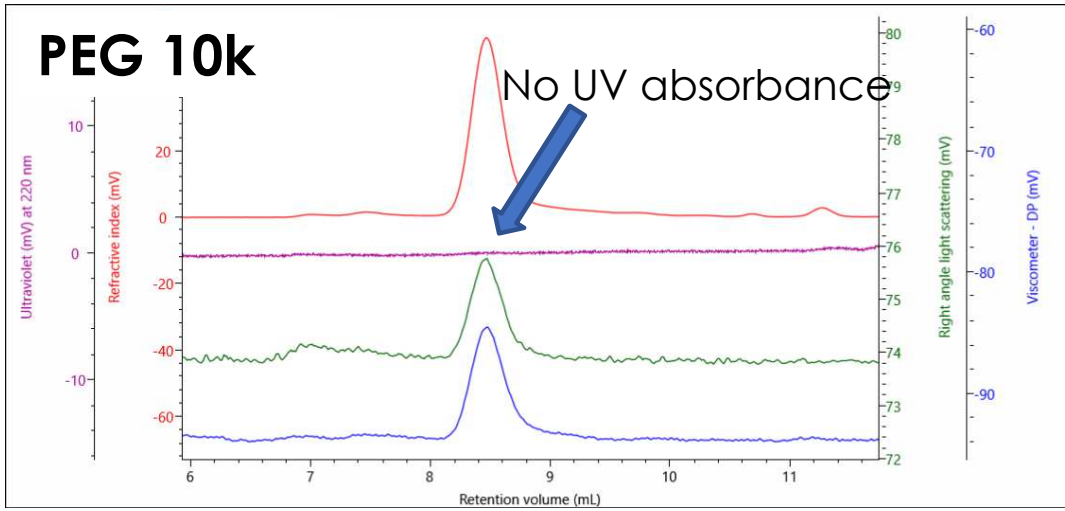
- In this study

OMNISEC set-up	
Column	Zenix-C 300
Flow rate	0.6 mL/min
Mobile phase	PBS
Column Temperature	30°C
Detectors Temperature	30°C
Autosampler Temperature	4°C
Injection volume	30 µL
Sample concentration	~0.3 mg/mL

Asparaginase & PEG starting materials

- Multi-detector chromatogram - pure components used for the PEGylated protein production.





	Weight- & number-average molecular weight		Dispersity	Intrinsic Viscosity	Hydrodynamic radius
Sample	**M_w (Da)**	**M_n (Da)**	**Đ**	**IV (dL/g)**	**Rh (nm)**
Asparaginase (tetramer peak)	134,000	133,600	1.003	0.024	3.69
PEG 10k	12,000	11,800	1.015	0.19	3.29

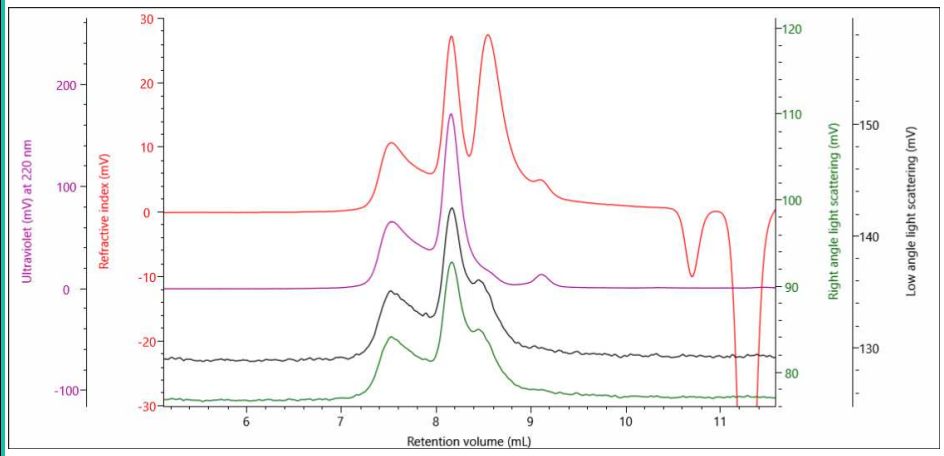
Monomeric Asparaginase M_w 33,500 Da

PEGylated L-Asparaginase (A-PEGy 10k)

- A-PEGy 10k – Standard reaction conditions

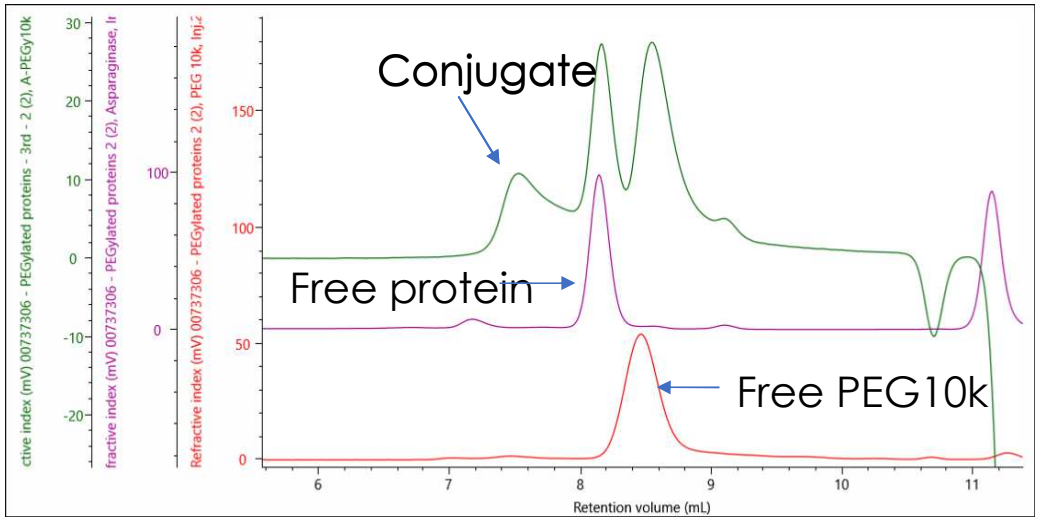
Sample	Equivalents of PEG	Time	pH	Concentration (mg/mL)
A-PEGy 10k	2.5	overnight	5.0	0.3

A-PEGy 10k – Multi-detector chromatogram



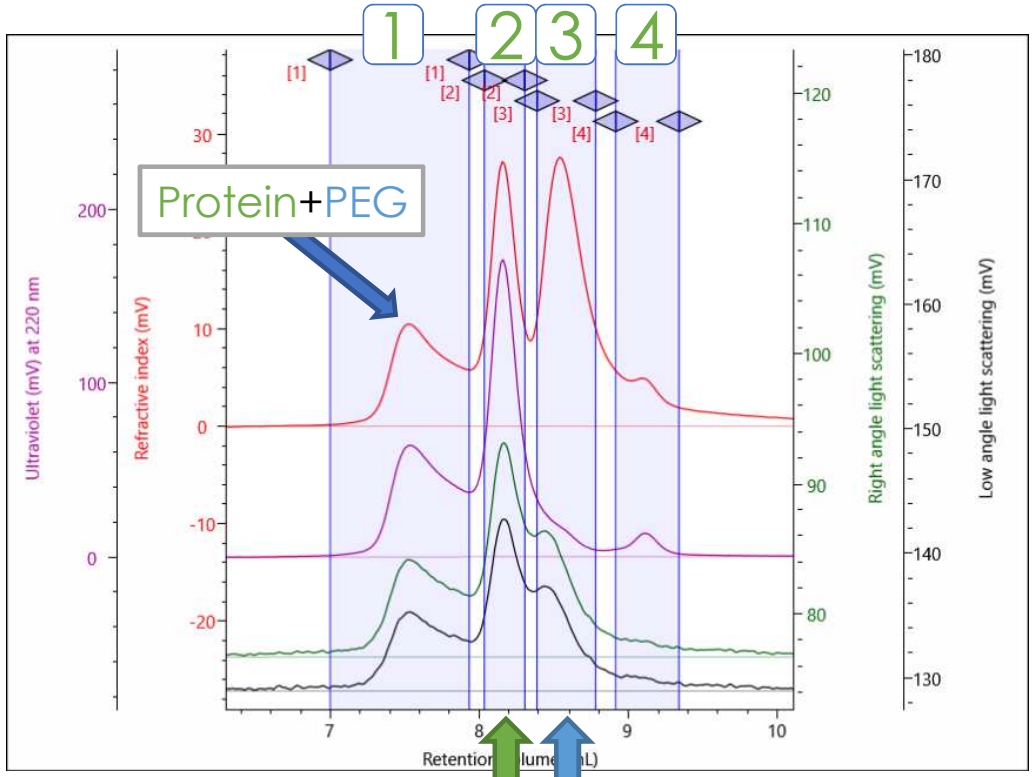
- Multiple peaks chromatogram
- Different detectors responses
- Where is the PEGylated protein?

RI Overlay – Conjugate/Free Protein/Free PEG10k



PEGylated L-Asparaginase

- A-PEGy 10k



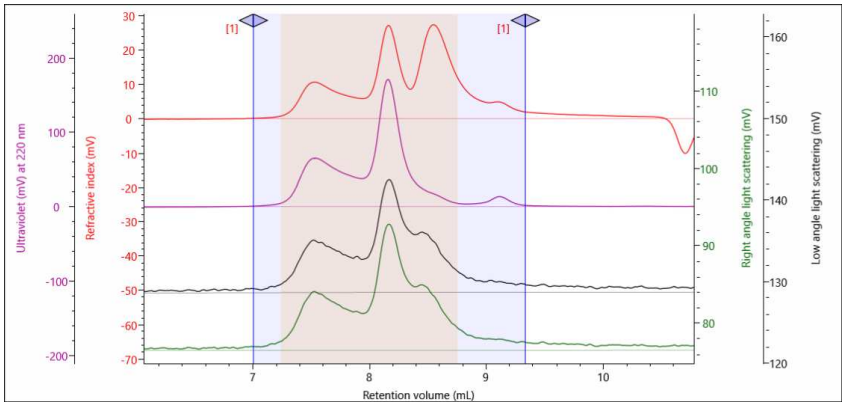
- Compositional analysis confirms the peaks corresponding to *unreacted* protein and PEG
- Concentration of components can be calculated

Sample	Replicates	RV (mL)	Weight Fraction PEG (%)	Weight Fraction Protein (%)	Concentration (Protein) (mg/mL)
Peak 1	i	7.5	12.5	87.5	0.51
	ii	7.5	12.7	87.3	0.51
	Average	**7.5**	**12.6**	**87.4**	**0.51**
Peak 2	i	8.2	8.1	91.9	0.53
	ii	8.2	8.2	91.8	0.54
	Average	**8.2**	**8.2**	**91.8**	**0.54**
Peak 3	i	8.6	91.5	8.5	0.05
	ii	8.6	91.4	8.7	0.05
	Average	**8.6**	**91.4**	**8.6**	**0.05**
Peak 4	i	8.9	78.8	21.2	0.12
	ii	8.9	79.2	20.8	0.12
	Average	**8.9**	**79.0**	**21.0**	**0.12**

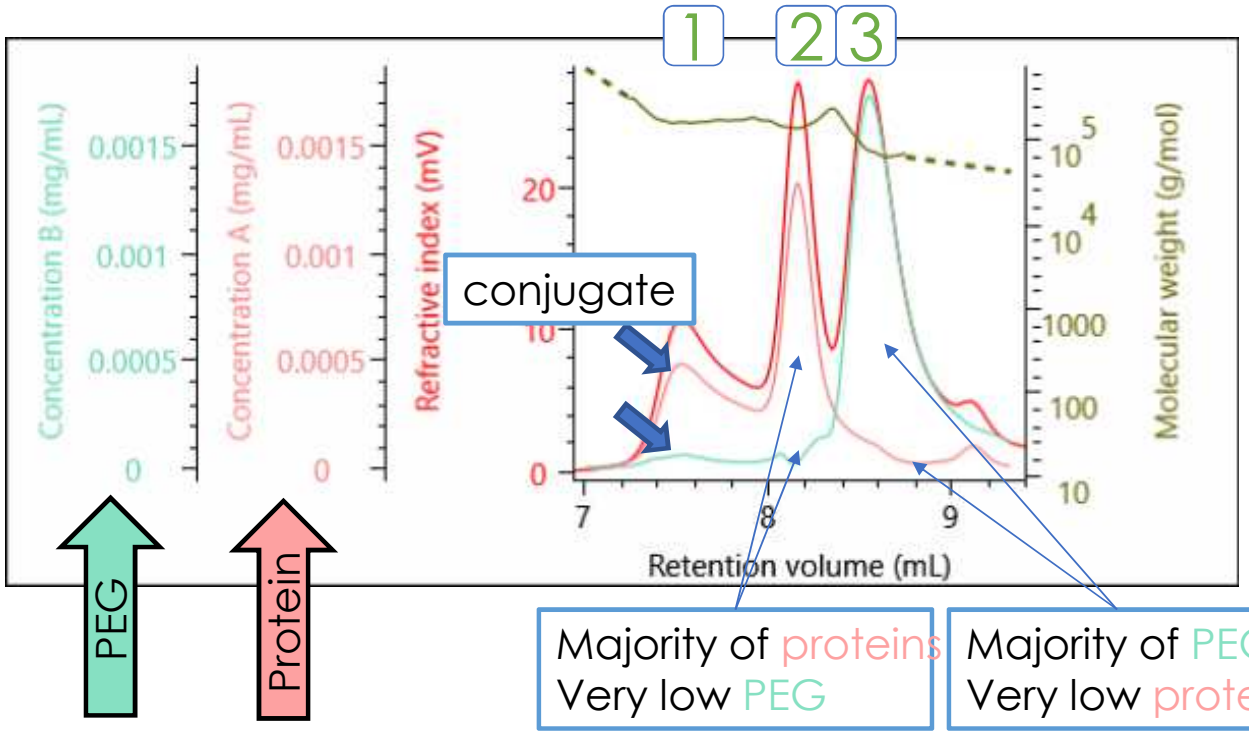
Concentration profiles

- A-PEGy 10k

- OMNISEC™ software allows you to look at the concentration profiles of protein and PEG by including the entire sample's chromatogram in the limits



Setting only one single set of limits



In conclusion

The OMNISEC is a multi detector SEC platform:

- Absolute Mw
- Oligomeric state
- Protein coniugation

Thank you for your attention

Any Question?

We are available to measure your samples:

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