The Field-Flow Fractionation Platform

POSTNOVA

Field-Flow Fractionation of Macromolecules and Structures That Cannot be Characterized by Conventional GPC/SEC Techniques

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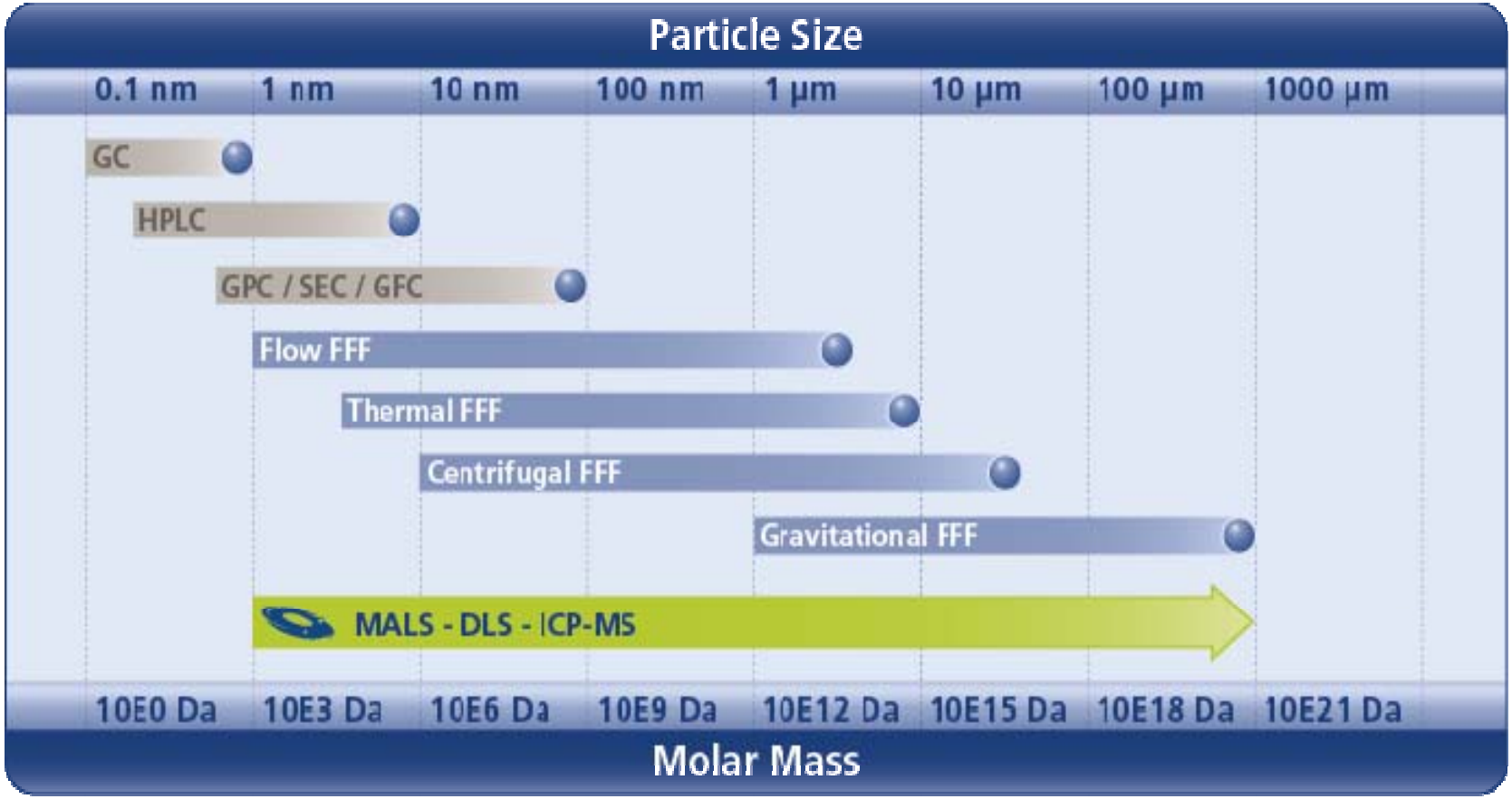
Advanced Characterization of Proteins, Biopolymers and Nanoparticles

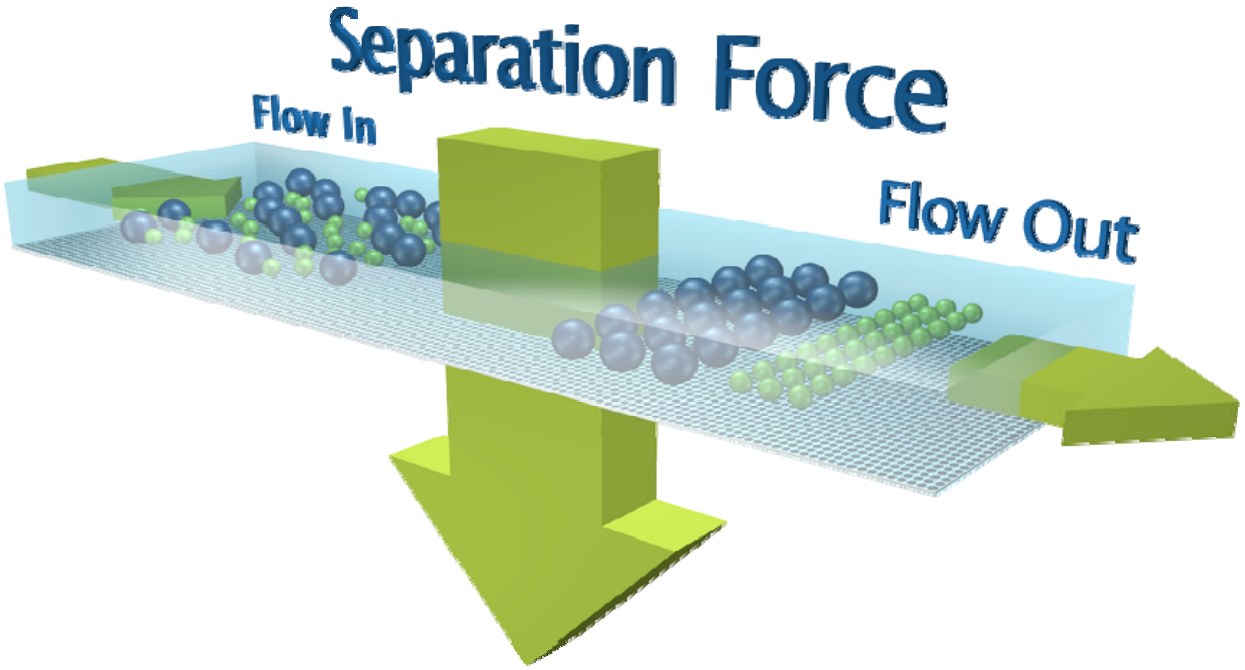
FFF Separation Range

Comparison Chromatography vs. Field-Flow Fractionation



FFF Principle

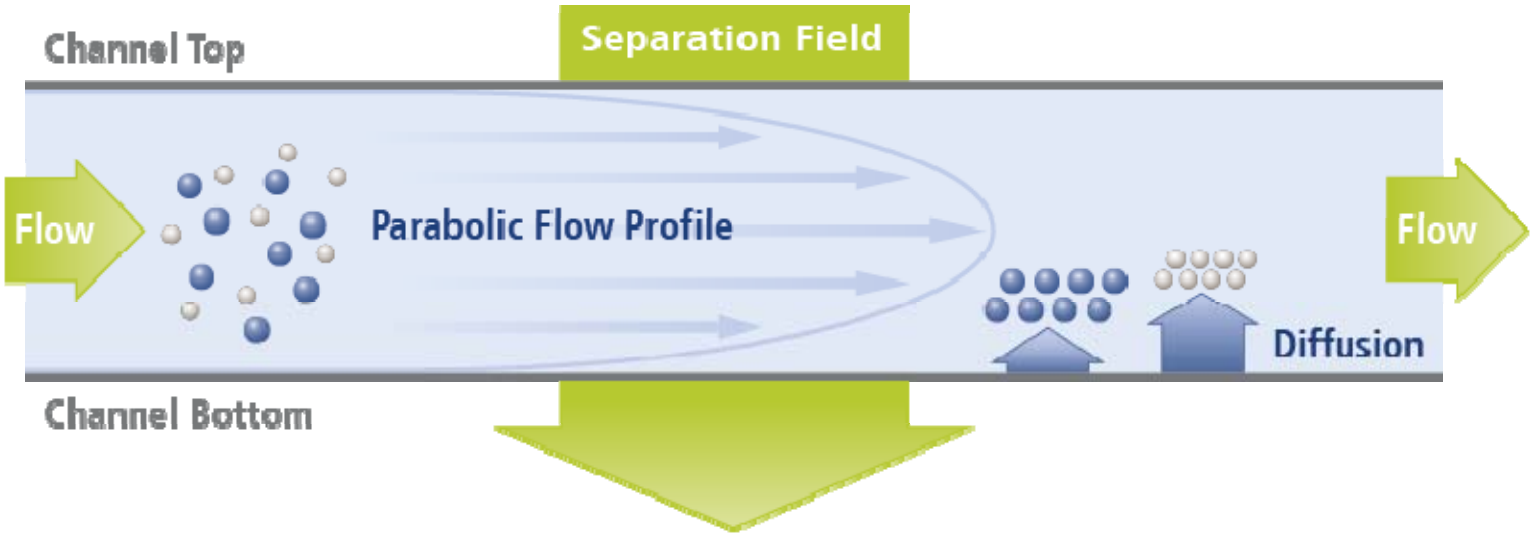
Separation Mechanism



- Separation in a narrow ribbon-like channel
- Laminar flow inside the channel
- External field perpendicular to the solvent flow

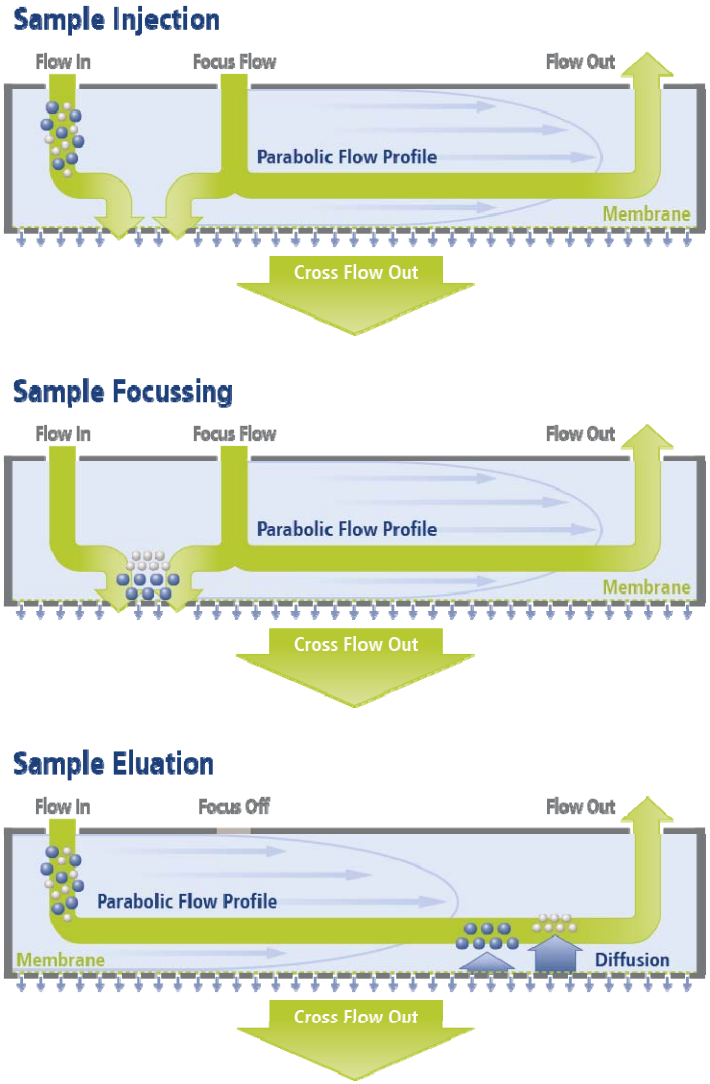
FFF Principle

Separation Mechanism



- Centrifugal Field
- Flow Field
- Thermal Field
- Gravitational Field

Asymmetric Flow FFF - Principle



Step 1

- Sample is injected directly into the flow stream
- A second pump provides focus flow
- Cross flow is achieved by a precise syringe pump
- Constant flow to the detectors, no valves

Step 2

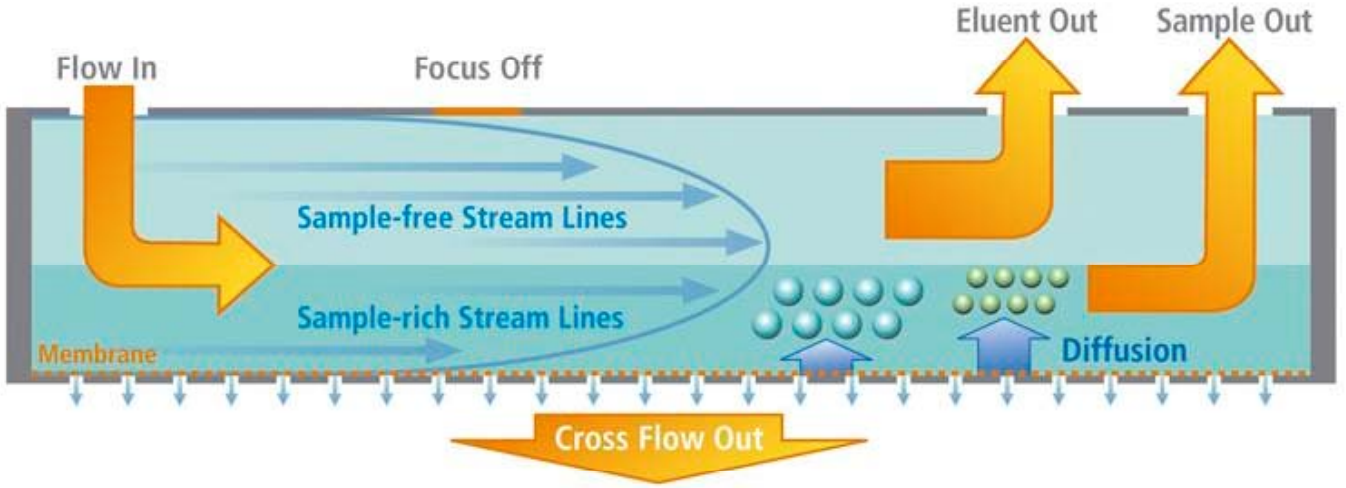
- Sample is focused to narrow band
- Improved resolution and sample washing
- Enrichment of very low concentrations

Step 3

- Focus flow stops and main pump elutes the sample
- Variable cross flow
- Detector flow stays constant

Asymmetric Flow FFF

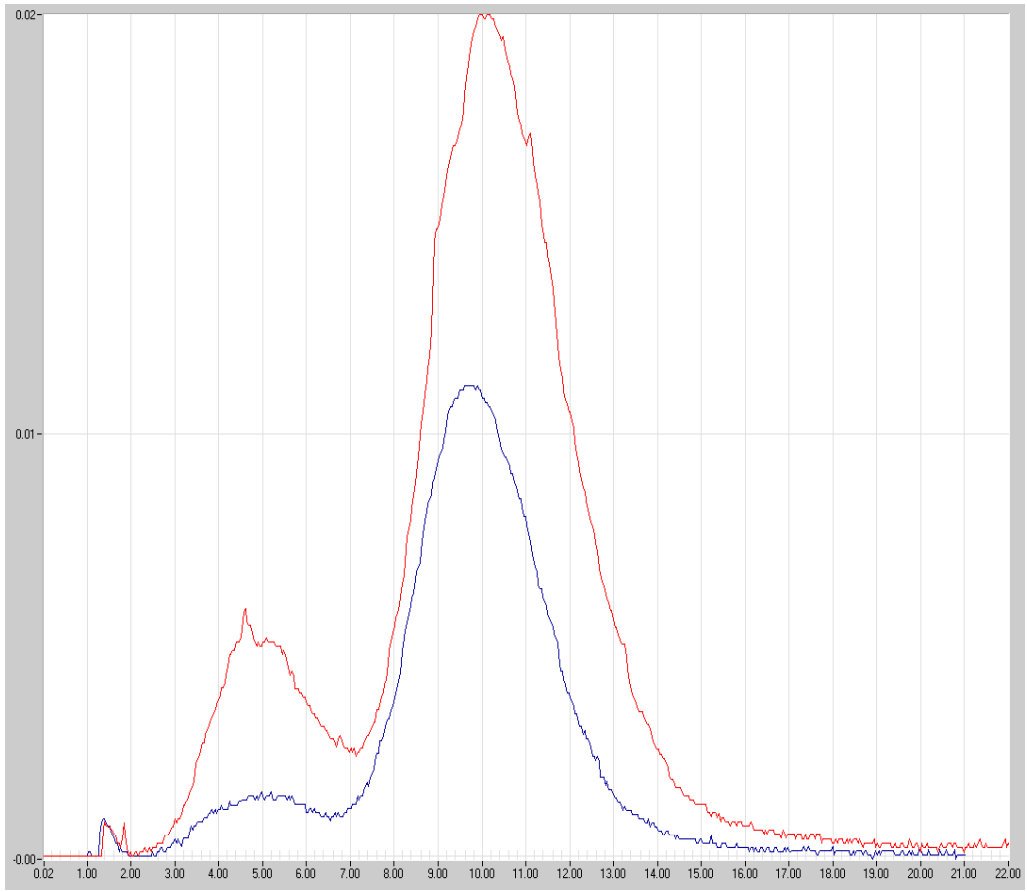
Smart Stream Splitting



- Major portion of flow in the channel is containing no sample
- Minor portions of the flow is carrying the sample above the membrane
- Splitting of those two stream via 2 outlets at the channel end
- Only the sample containing sub-stream is guided to the detector
- Sample dilution is reduced at channel outlet
- Increase of sensitivity by factor 5 or more depending on conditions

Smart Stream Splitting

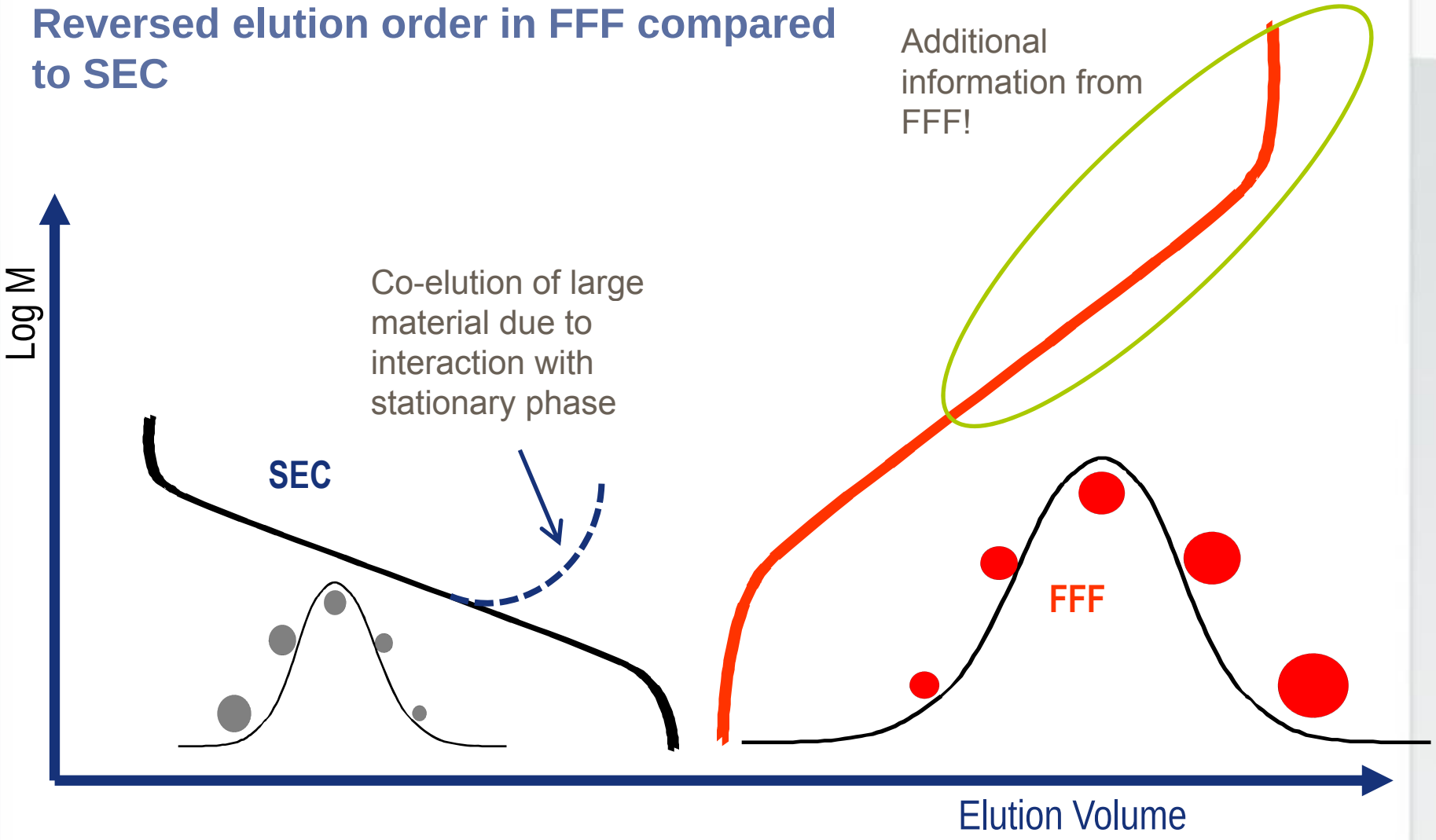
HDL Lipoprotein Preparation for Mass Spectroscopy



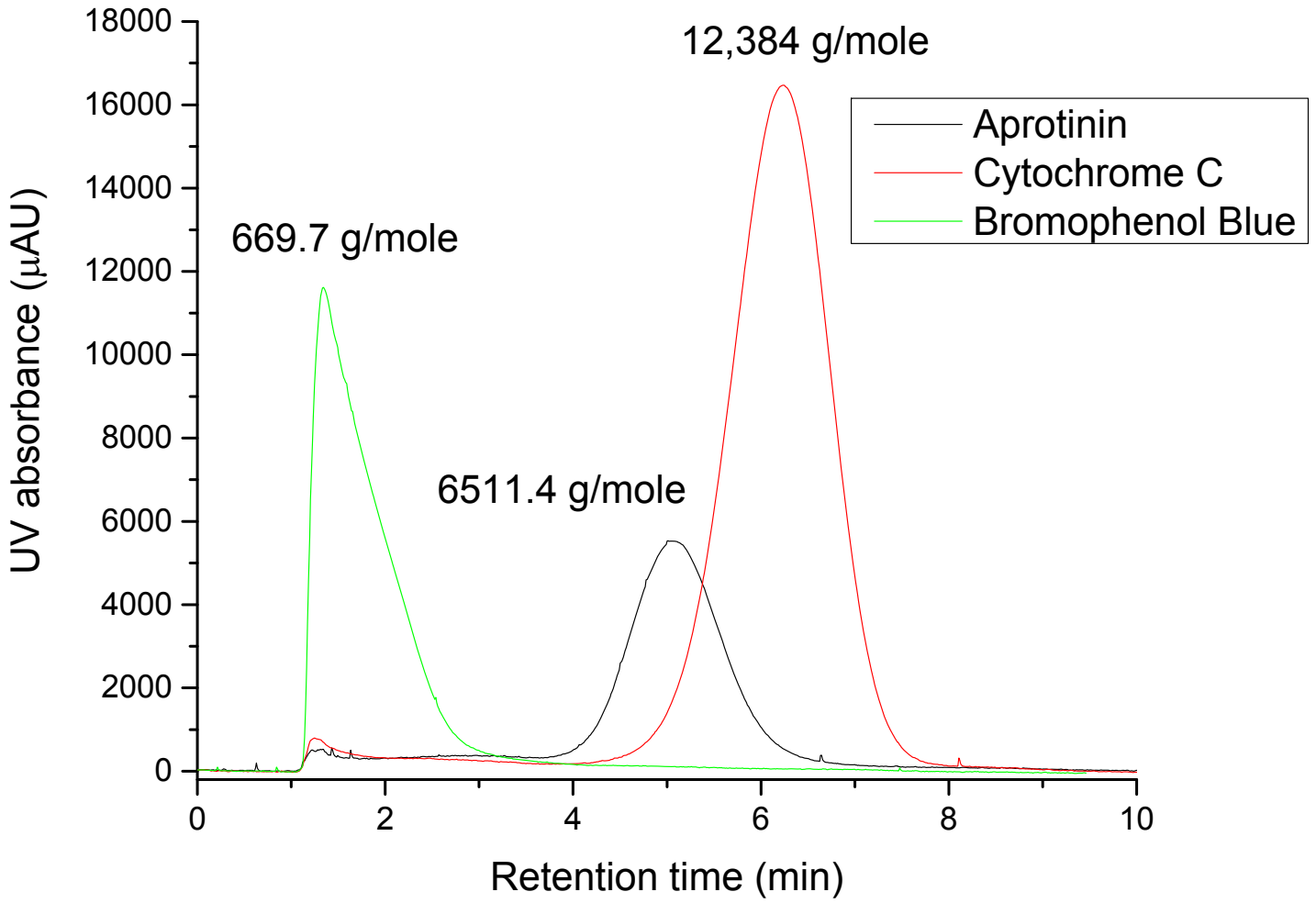
Enrichment of HDL by a factor of 2 using Smart Stream Splitting

FFF versus SEC

Reversed elution order in FFF compared to SEC



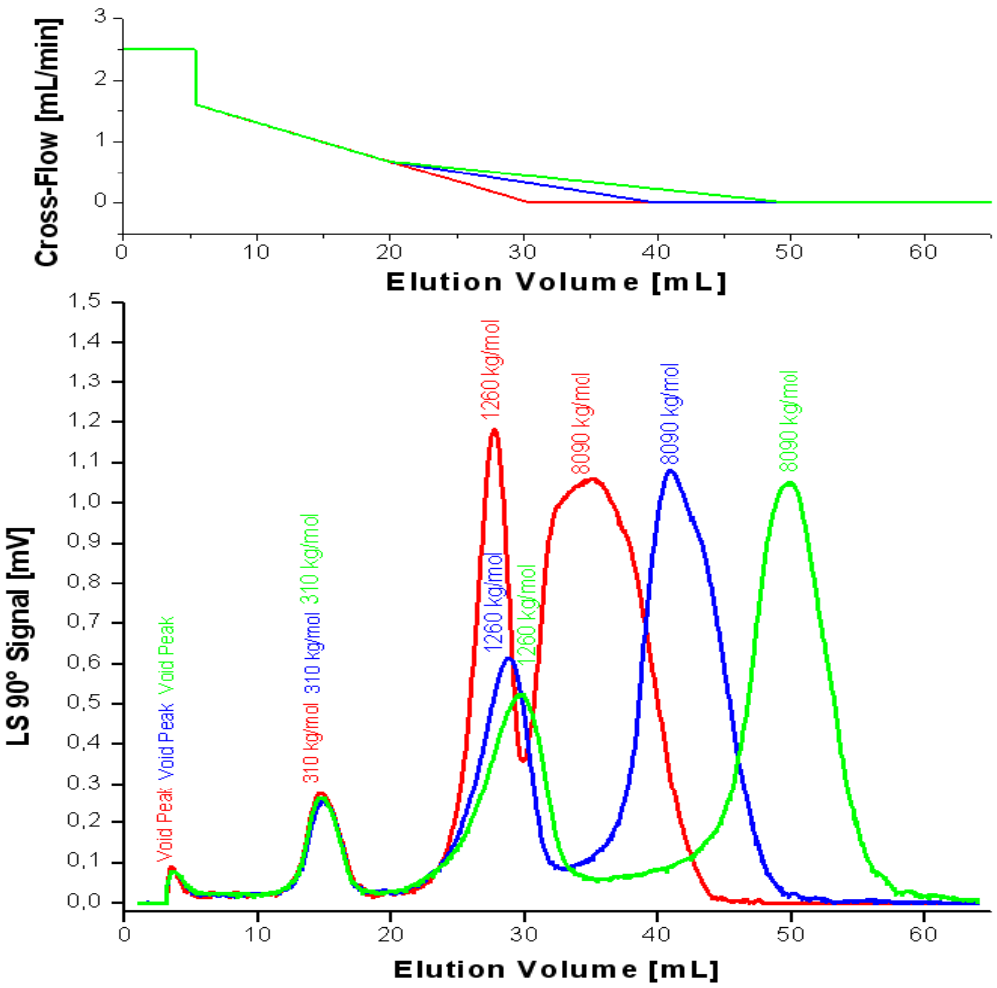
How Low Can a Postnova AF4 go!!



Flexibility of the Cross-Flow Gradient

“Tailor-made” separation in AF4 realized by cross-flow-adjustment

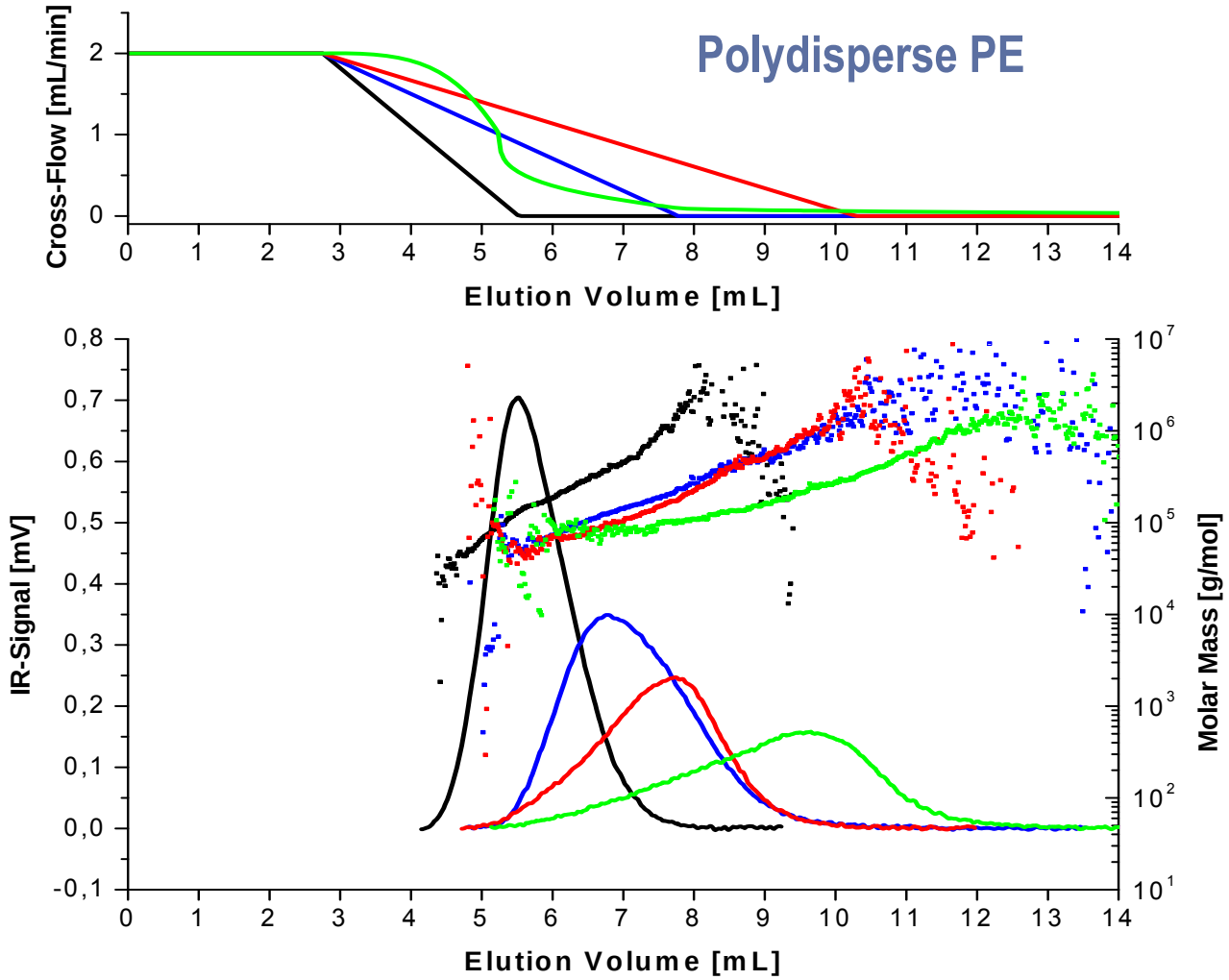
- Extension of Cross-Flow causes better separation
- Selective enhancement of separation
- In SEC not possible!
  - → Column change is time consuming and expensive
  - → Calibration of column determines separation



Mixture of narrow PS: 300, 1200 & 8000 kg/mol

Flexibility of the Cross-Flow Gradient

- Gradient of any shape can be used
- Adjustment of separation according to special requirements of the sample



System Information

Injector	PN5300 Auto Injector
Separator	AF2000 MF Flow FFF System (AF4)
Channel	AF2000 Analytical Channel (AF4) 350 µm Thickness; NovaRC 10kDa Membrane
Detection	PN3241 UV – Absorbance, 254 nm PN3150 RI – Refractive Index PN3621 MALS – Static Light Scattering, 532 nm
Fraction.	n.a.
Eluent	Sample UW001: 0.1 M NaCl + 0.2 g/L NaN_3 Eluent filtered by 0.1 µm filter Sample UW002: THF
Flow Rate	Sample UW001: 0.5 mL/min and 0.5 mL/min **Smart Stream Splitting** Sample UW002: 0.5 mL/min

Postnova AF2000 Multi Flow FFF

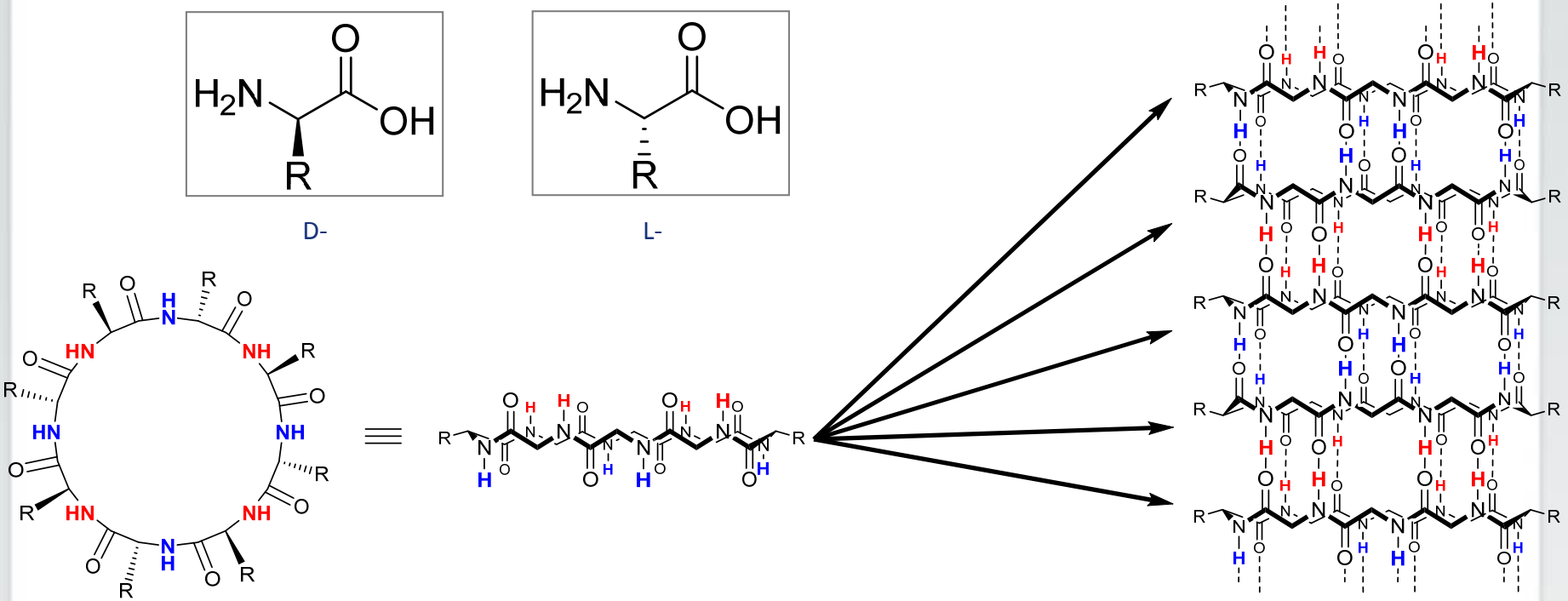
Summary (AT THE BEGINNING)

The best fit of the shape (SAS view) doesn't agree with the fit of the intensity

Results of SLS agree with the SANS results in intensity

The hypothesis: Most of the system is composed of unimers with a small amount of very long cylinders that explain both the results of SLS and SANS was confirmed by AF4 results

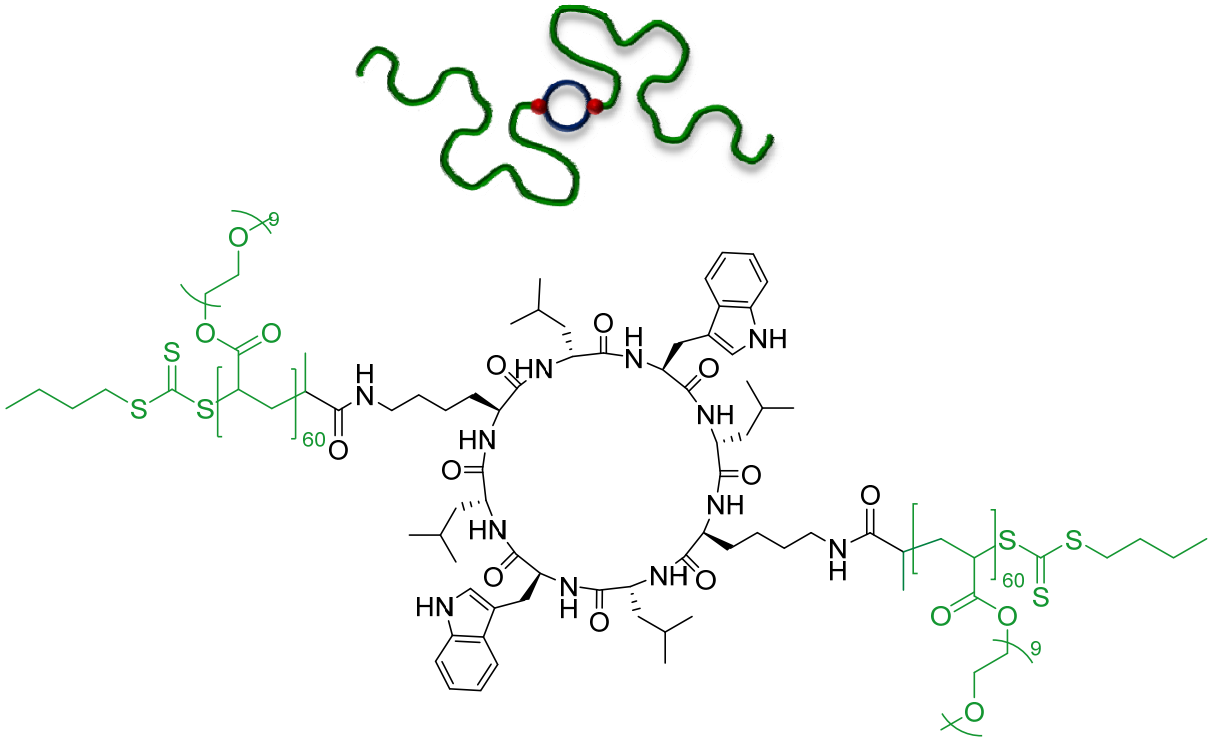
β-Sheet Forming Cyclic Peptide Nanotubes



Ghadiri, M. R.; Granja, et al., *Nature*, ***1993, 366 (6453), 324.***
Horne, W. S., *et al., Bioorg. & Med. Chem.* ***2005, 13 (17), 5145.***

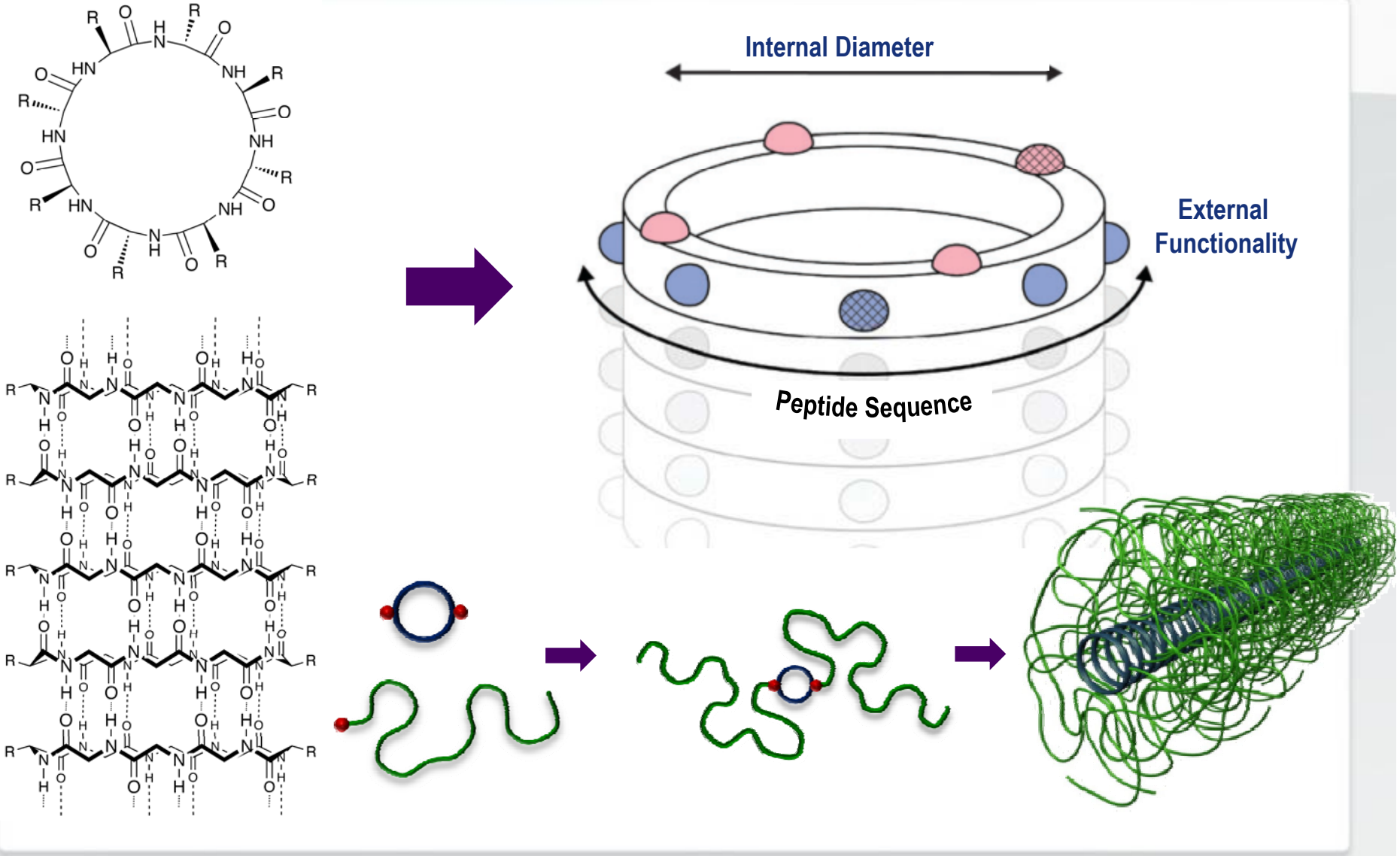
SANS

Study of SL155



$PEGA_{60}$ DP = 60

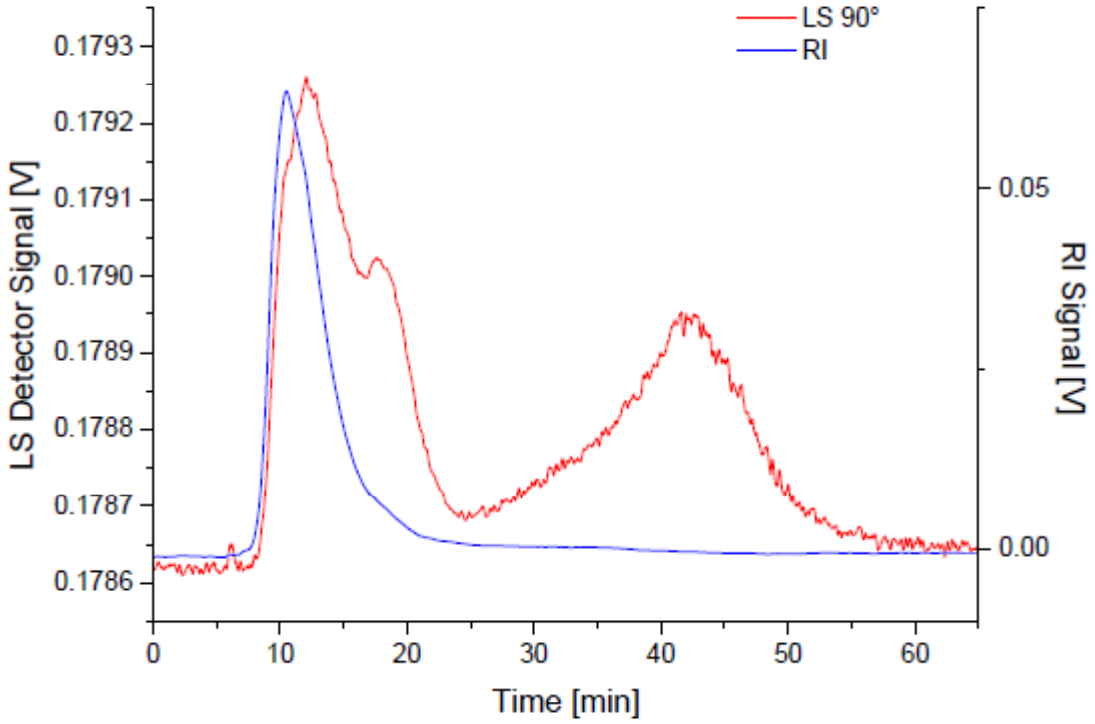
Nanotube Design Elements



Chapman, R., *et al.*, *Chem. Soc. Rev.* **2012, *41, 6023.***
Couet, J.; Biesalski, M., *Small* ***2008, 4 (7), 1008.***

Sample UW001: SL155

Raw Data Fractogram of AF4 - MALS and UV



- The 1st peak shows a double structure and was detected between 8 and 25 min by light scattering detection.
- A 2nd peak was detected between 25 and 60 min by light scattering detection.

System

- PN5300 Auto Injector
- AF2000 FFF System
- PN1650 Smart Stream Splitter
- PN3621 MALS Detector
- PN3241 UV Detector
- PN3150 RI Detector

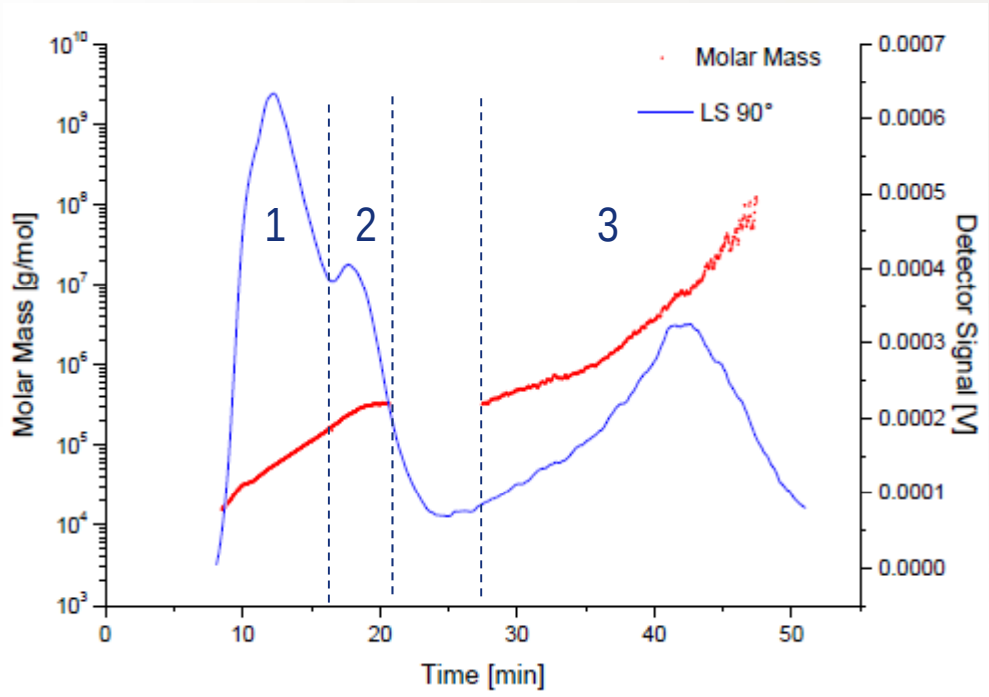
Conditions

- Injection Volume: 20 µL
- Concentration: 10 mg/mL
- LS 90°(red trace)
- RI Detector (blue trace)

Sample UW001: SL155

Overlay: Molar Mass and LS Signal

- The Molar Mass was calculated from MALS and RI data
- given value for dn/dc = 0.12 mL/g
- In the 1st Fraction the Sample contains Material with a Molar Mass of appr. 5.4 x 10^4 g/mol (w-Average), in the 2nd Fraction of 2.6 x 10^5 g/mol and in the 3rd Fraction of 2.3 x 10^6 g/mol.



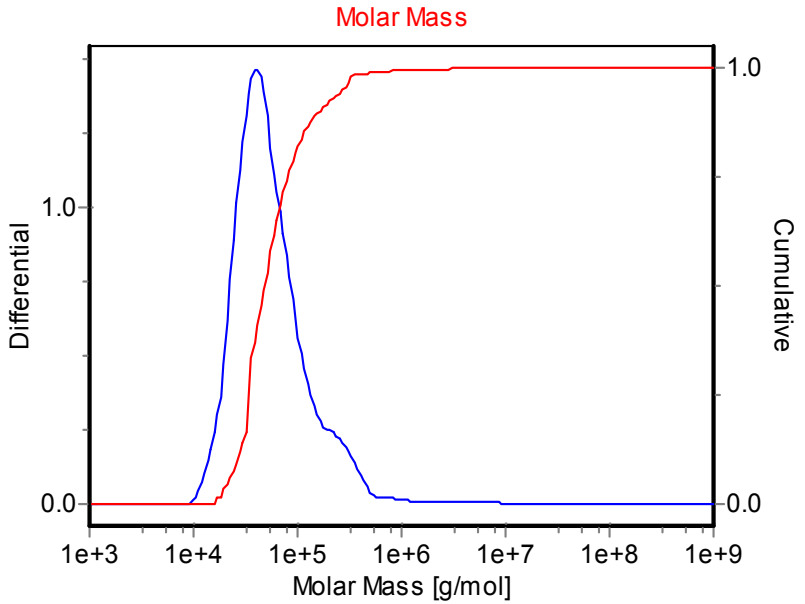
Conditions

- Molar Mass (red dots)
- LS Signal (blue trace)
- Fitting by Random Coil Model

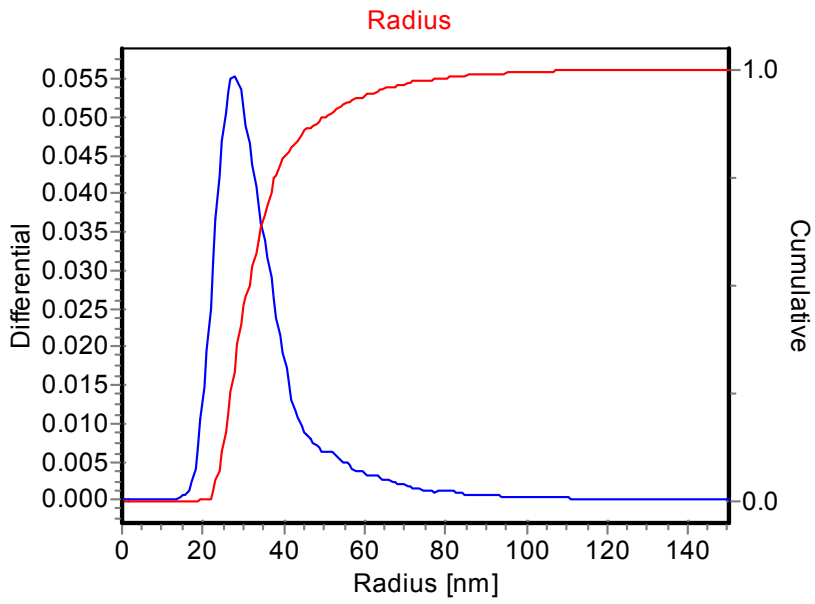
		M_w [g/mol]
1. Fraction 8.5 – 16.5 min	*n*-Average	4.2 x 10^4
	***w*-Average**	**5.4 x 10^4**
	z-Average	7.2 x 10^4
2. Fraction 16.5 – 20.8 min	*n*-Average	2.5 x 10^5
	***w*-Average**	**2.6 x 10^5**
	z-Average	2.7 x 10^5
3. Fraction 27.5 – 47.5 min	*n*-Average	7.1 x 10^5
	***w*-Average**	**2.3 x 10^6**
	z-Average	1.9 x 10^7

Sample UW001: SL155

Molar Mass Distribution



Particle Size Distribution



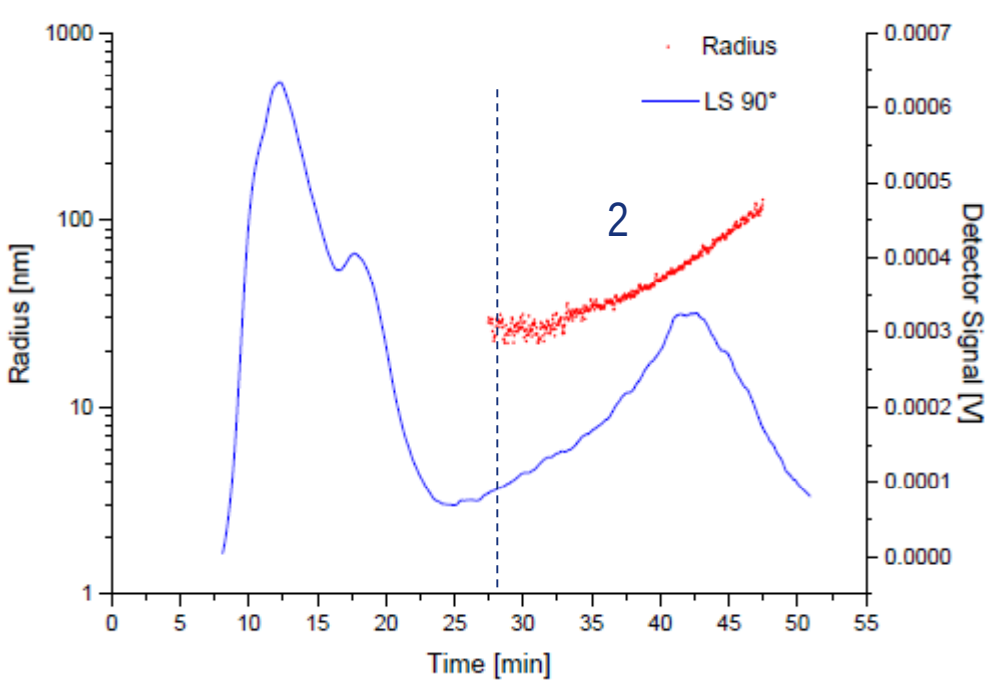
Differential Molar Mass/Particle Size Distribution (blue trace), Cumulative Molar Mass/Particle Size Distribution (red trace)

The sample shows a multimodal distribution. For the 1st fraction (8.5 – 16.5 min) the distribution is in the range of 1.5 x 10^4 – 1.7 x 10^5 g/mol, for the 2nd fraction (16.5 – 20.8 min) in the range of 1.7 x 10^5 – 3.4 x 10^5 g/mol and for the 3rd fraction (27.5 – 47.5 min) in the range of 3.4 x 10^5 – 1.2 x 10^8 g/mol.

Sample UW001: SL155

Overlay: Radius of Gyration and LS Signal

- The Radius of Gyration was calculated from MALS angular data
- The 3rd Fraction shows a Radius of Gyration of 65 nm (*z*-average)



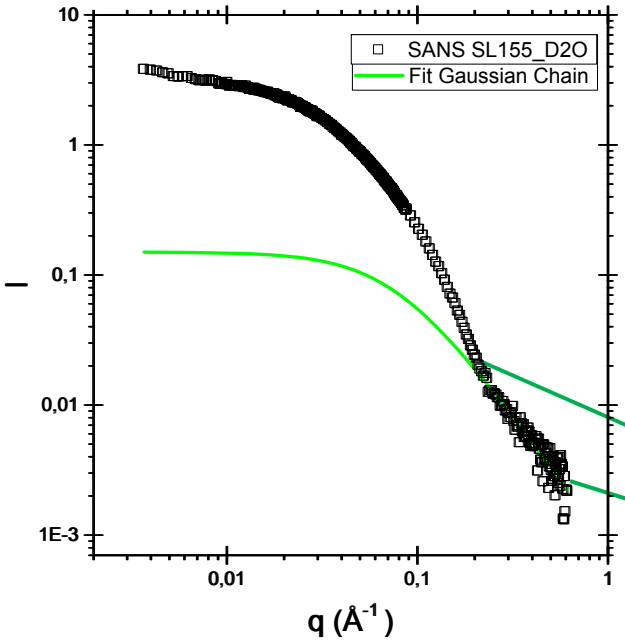
Conditions

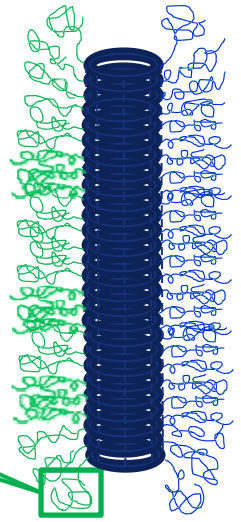
- Radius of Gyration (red dots)
- LS Signal (blue trace)
- Fitting by Random Coil Model

		R_g [nm]
3. Fraction 27.5 – 47.5 min	*n*-Average	29
	w-Average	35
	***z*-Average**	**65**

Study of SL155 in D_2O 10g/L

- Using the softare SAS view it's possible to fit the shape
- of the data to have an idea of the shape of the objects in solution

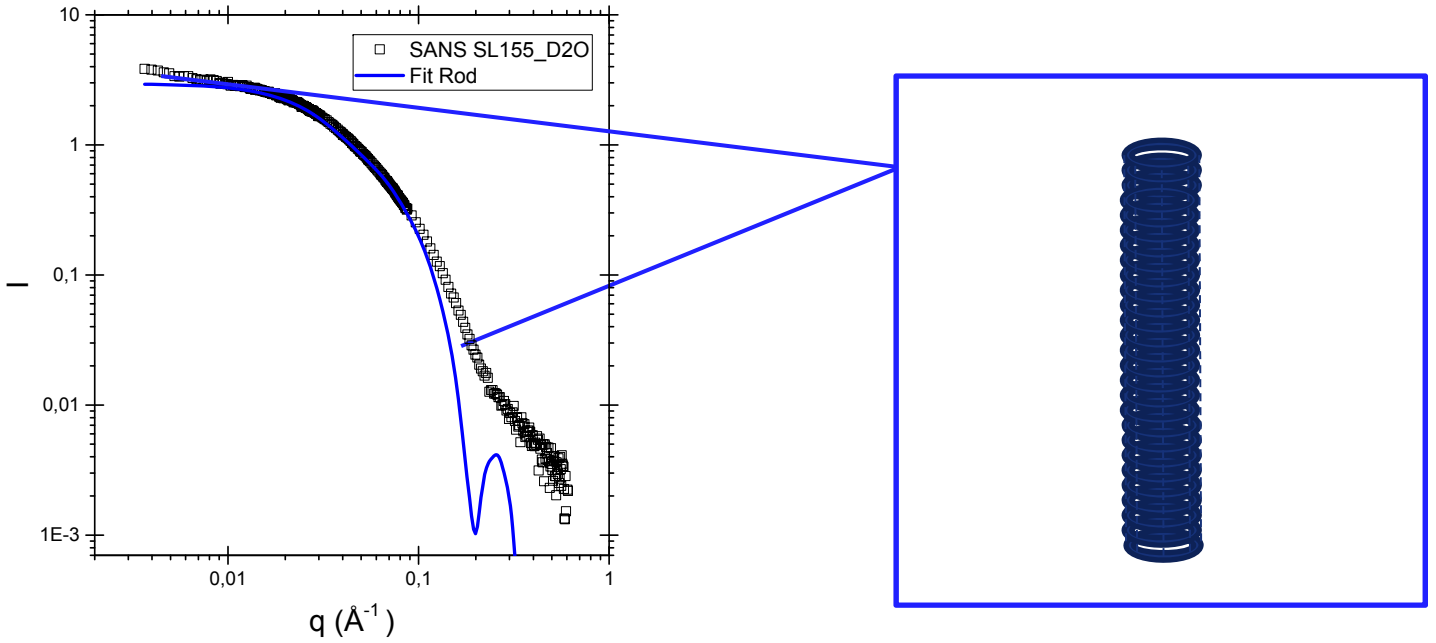




- The tail at high q is well fitted with a Gaussian chain form factor

Study of SL155 in D_2O 10g/L

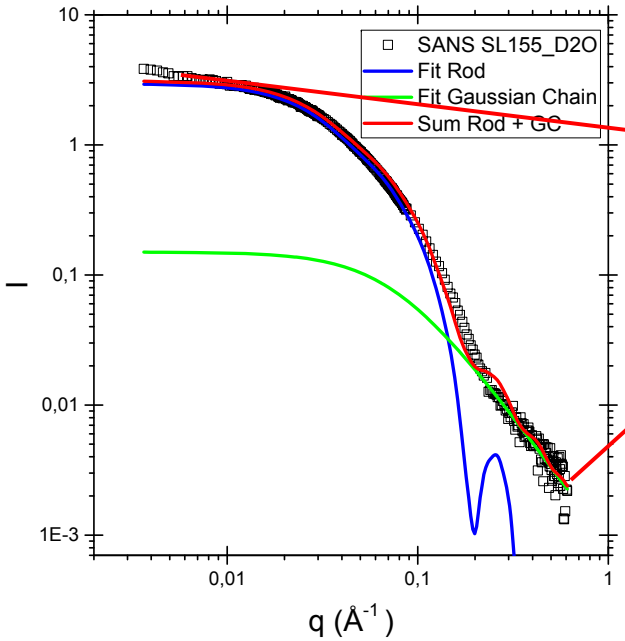
- Using the software SAS view it's possible to fit the shape of the data
- to have an idea of the shape of the object in solution

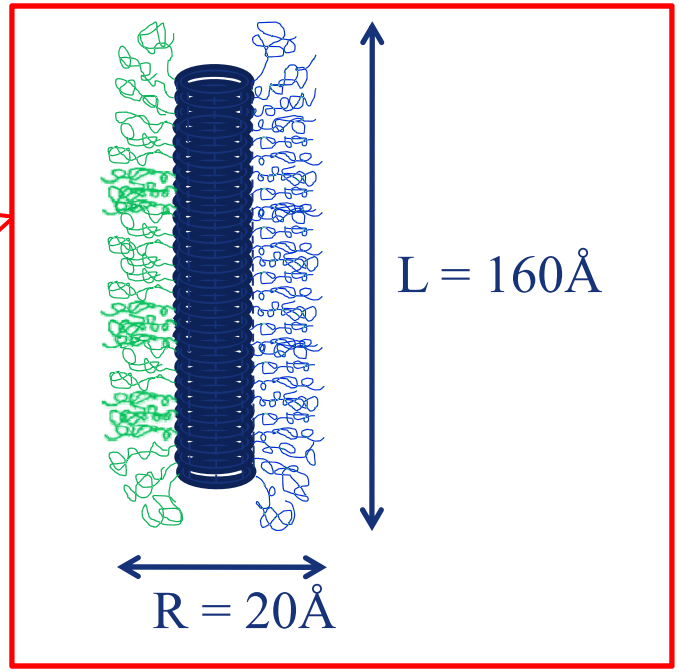


- The rollover is well fitted by a Rod form factor

Study of SL155 in D_2O 10g/L

➢ Using the software SASit's possible to fit the shape of the data to have an idea of the shape of the objects in solution

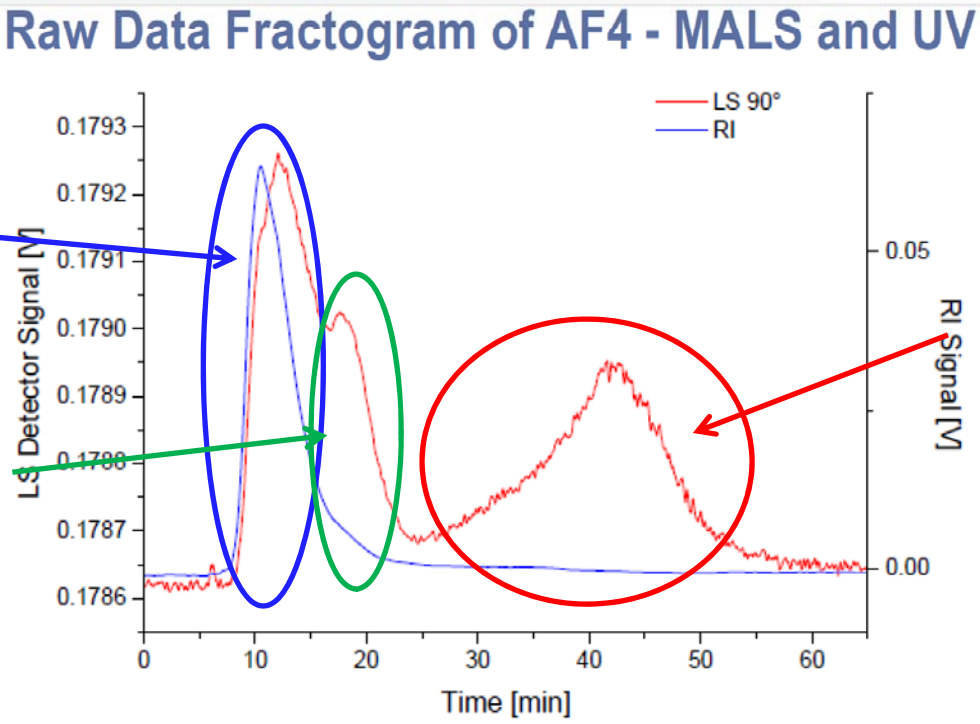




➢ A sum of Rod and Gaussian Chain form factors fit nicely all the q range

➢ L = 160Å => Nagg = 32 (Number of Aggregates)

AF4 analysis of SL 155 in water



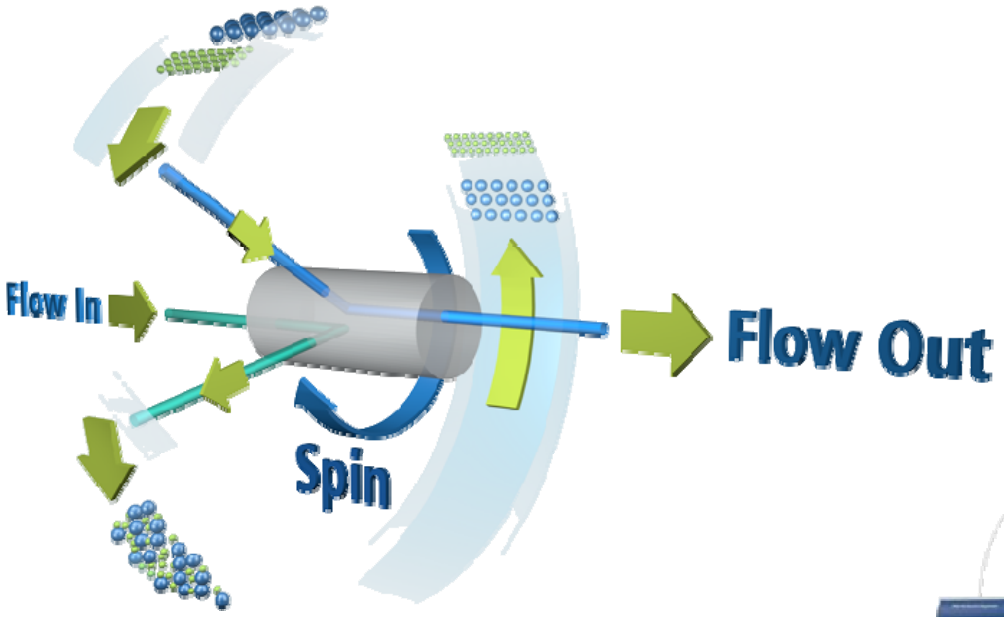
- Main population
  Mw = 54000g/mol
  (unimers)
- Second population
  Mw = 2.6×10^5g/mol
  Nagg = 4
- Minor population
  Mw = 2.3×10^6g/mol
  Nagg = 38.7

- AF4 results agree with SLS and SANS results: unimers are the main population
- An additional population of Nagg = 4 that was not observed with SLS or SANS is observed
- The minor population (Nagg = 38.7) explain the result in shape of SANS

Centrifugal FFF

Centrifugal FFF – Principle

Separation Principle



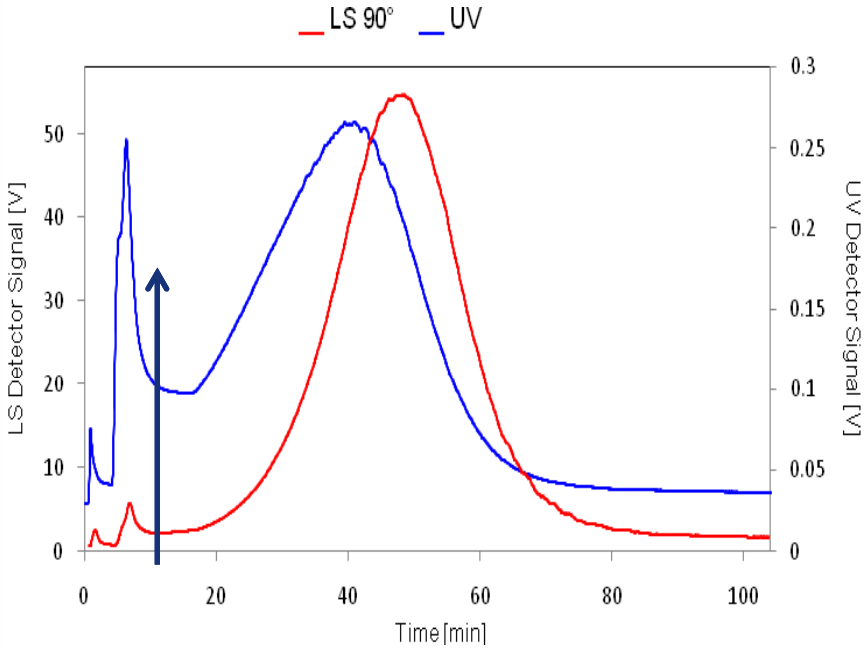
Content

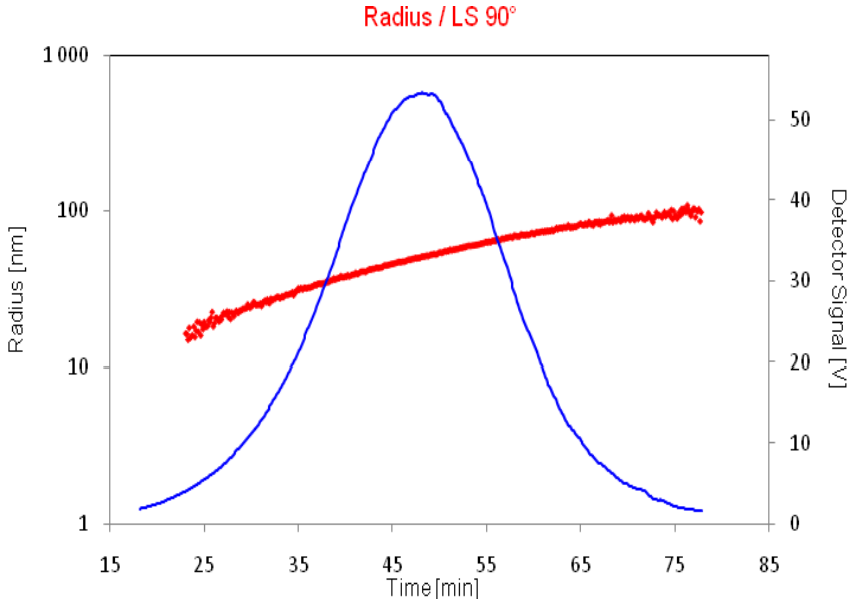
- FFF Principle
- Systems
- Applications
- Summary

- Gravity Separation Field up to 2.500 g
- Size Separation Range: Particles 5 nm – 100 µm
- Separation based on Size and Density

Polyelectrolyte Encapsulated Around NanoParticle

Shown are the raw data signals of the Light Scattering detector at 90° (red line) and the UV detector at 254 nm using Centrifugal Field Flow Fractionation CF3

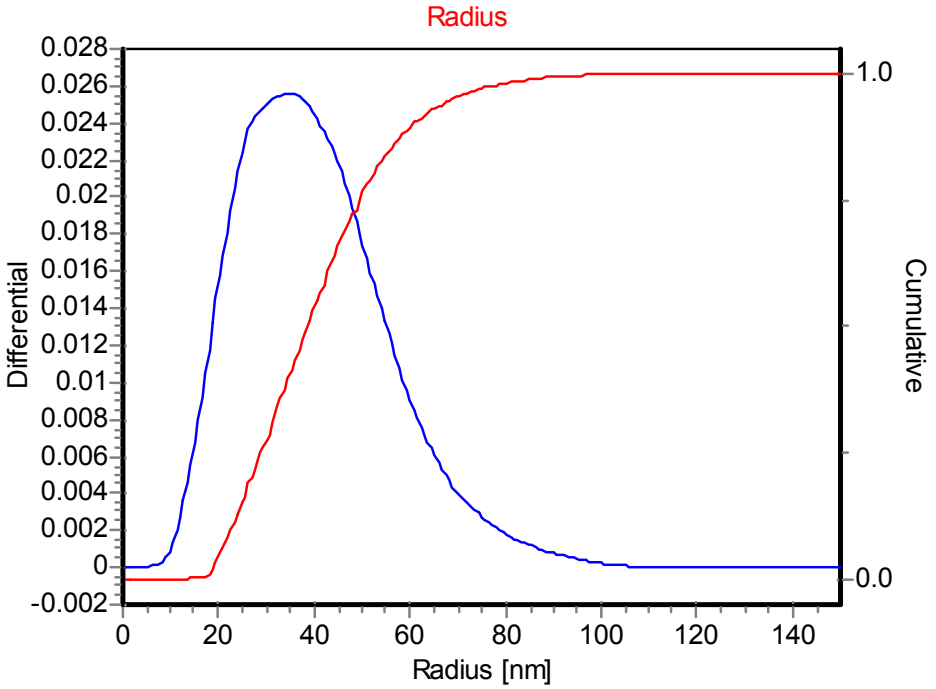




Using Centrifugal FFF (CF3) we can separate the free Polyelectrolyte from the cross-linked encapsulated nanoparticle.

Encapsulated Nanoparticle with Cross-linked Polyelectrolyte Particle Distribution using CF3

In the following figures the particle size distribution is shown:
Differential Radius Distribution (blue line) and Cumulative Radius Distribution (red line) of the second peak of sample.

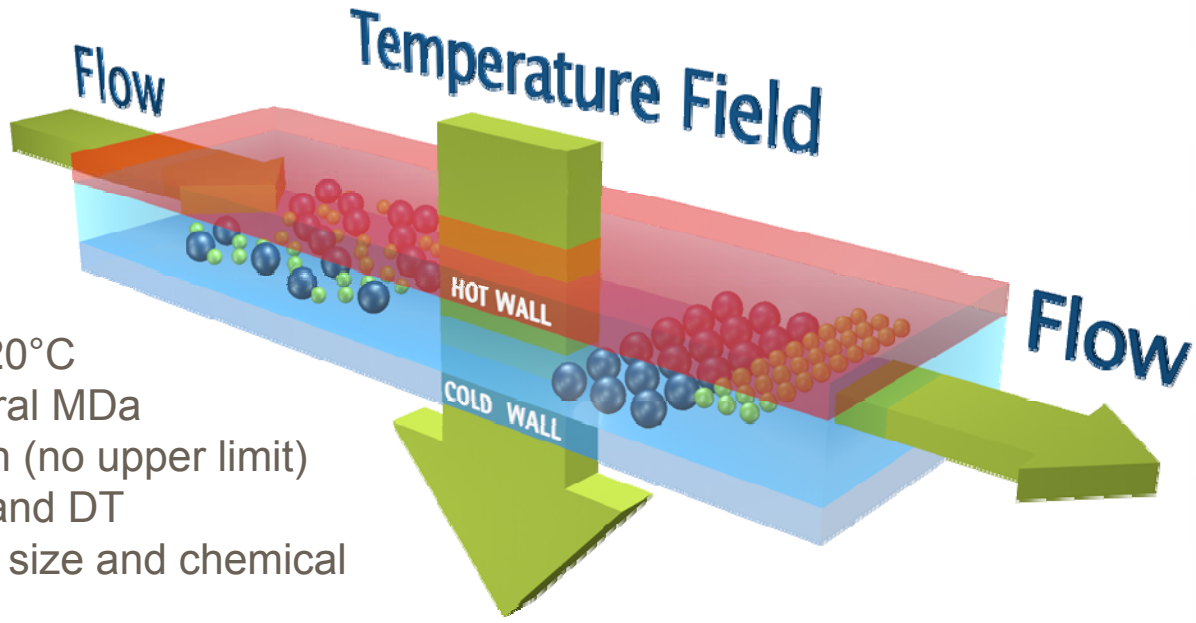


	Rg [nm]
n-Average	30
w-Average	41
z-Average	55

Thermal FFF

Thermal Field-Flow Fractionation

TF3 involves a hot and cold plate to generate a temperature gradient perpendicular to the separation channel. Thermal Diffusion occurs and this us often a property of the polymers morphology.



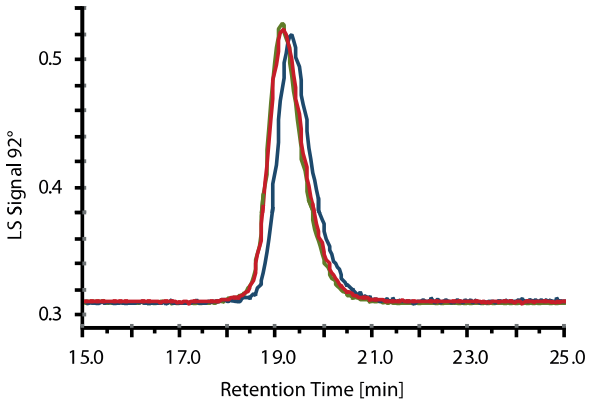
- Thermal gradient up to Δ120°C
- Separation kDa up to several MDa
- Analysis time, 10 – 120 min (no upper limit)
- Separation depends on D and DT
  → Separation according to size and chemical composition

SEC versus Thermal FFF

Analysis of PS and PMMA by SEC and Thermal FFF

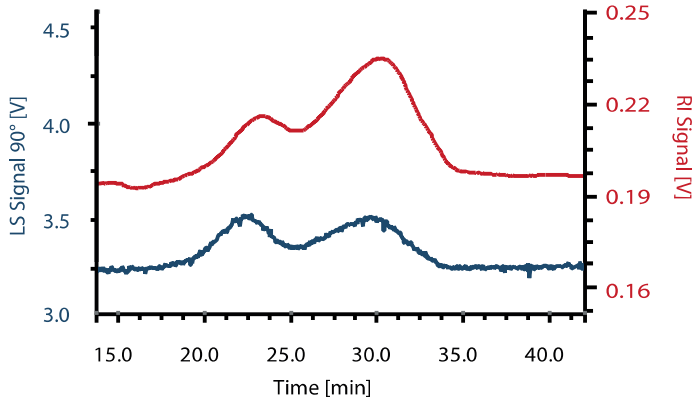
PS, PMMA and a mixture of both standards in THF. Taking advantage of the separation by chemical composition in TF3.

PS 96 kDa kg/mol
PMMA 106 kDa mix
PS 96 kDa - PMMA 106 kDa mix



SEC Chromatogram of PS, PMMA and Mixture

PS 96 kDa - PMMA 106 kDa mix



TF3 Fractogram showing RI and LS Signal of mixed PS-PMMA standards.(Δ T = 115 K).

Component	Retention Time (R_T)	Molecular Mass (M_w)
PS	19.1 - 24.7 min	95.7
PMMA	26.2 - 34.5 min	104.4

Conclusion

- **Field Flow Fractionation Complements SEC/GPC in standard separations**
- **FFF solves problems when the mass or particle size of the macromolecule exceeds the pore size of the column**
- **FFF solves the problem when the interaction between the macromolecule and the packing material limits the solvent and pH choice (GPC Symposium 2014 Presentation)**
- **FFF solves the problem when the macromolecule is reactive and unstable and the removal of packing and solvent control enables sensible elution characteristics**
- **FFF can be tailored to the application**
- **FFF is Great Fun to do in the lab ;) !!**