

Separation of Hyaluronic Acid on a Newly Developed Frit Inlet Asymmetrical Flow Field Flow Fractionation System (FI-AF4)

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Introduction

Hyaluronic acid (HA) is a naturally occurring linear polysaccharide consisting of D-glucuronic acid and N-acetyl-D-glucosamine. Generally, the molecular weight (MW) of naturally occurring HA is in the range 10⁵-10⁷ Da. In the human body, HA is mainly found in connective tissues, in particular in the synovial fluid (knee) where it primarily acts as a shock-absorbing and lubricating agent. HA's distinctive viscoelastic properties, coupled with its biocompatibility and resorbability have led to a wide range of biomedical and pharmaceutical applications.

HA is produced by Novozymes Biopharma by fermentation of the microorganism *Bacillus subtilis*, which is considered as safe host by the European Food Safety Authority. To achieve a high degree of purity, the process uses minimal media, no animal-derived raw materials and a proprietary water-based technology that eliminates the use of organic solvents.

HA physicochemical properties are largely governed by the mean MW of the polysaccharide as well as its molecular weight distribution (MWD), and analytical tools for characterizing these parameters are therefore of great importance. Size exclusion chromatography (SEC) has traditionally been employed to determine the MW and MWD of HA. However, SEC is often not well suited to characterize high MW HA due to limitations in pore sizes of commercially available column packing material, the possibility of shear-induced degradation of large molecules, a lack of appropriate HA standards needed for calibration and possible blocking of the column (cf. Figure 1).

Asymmetrical Flow Field Flow Fractionation (AF4) has been used as a complementary method for size separation and characterization of high-MW HA with online coupling of multi-angle light scattering (MALS) and refractive index (RI) detectors [1, 2, 3, 4]. But conventional AF4 is prone to produce HA aggregation during the focusing step leading to an increase of molar mass with increased sample concentration at the membrane (cf. Figure 2). In this work we show that Frit-Inlet AF4 [5] does not produce this artefact and is a preferred FFF method for the separation of HA molecules.

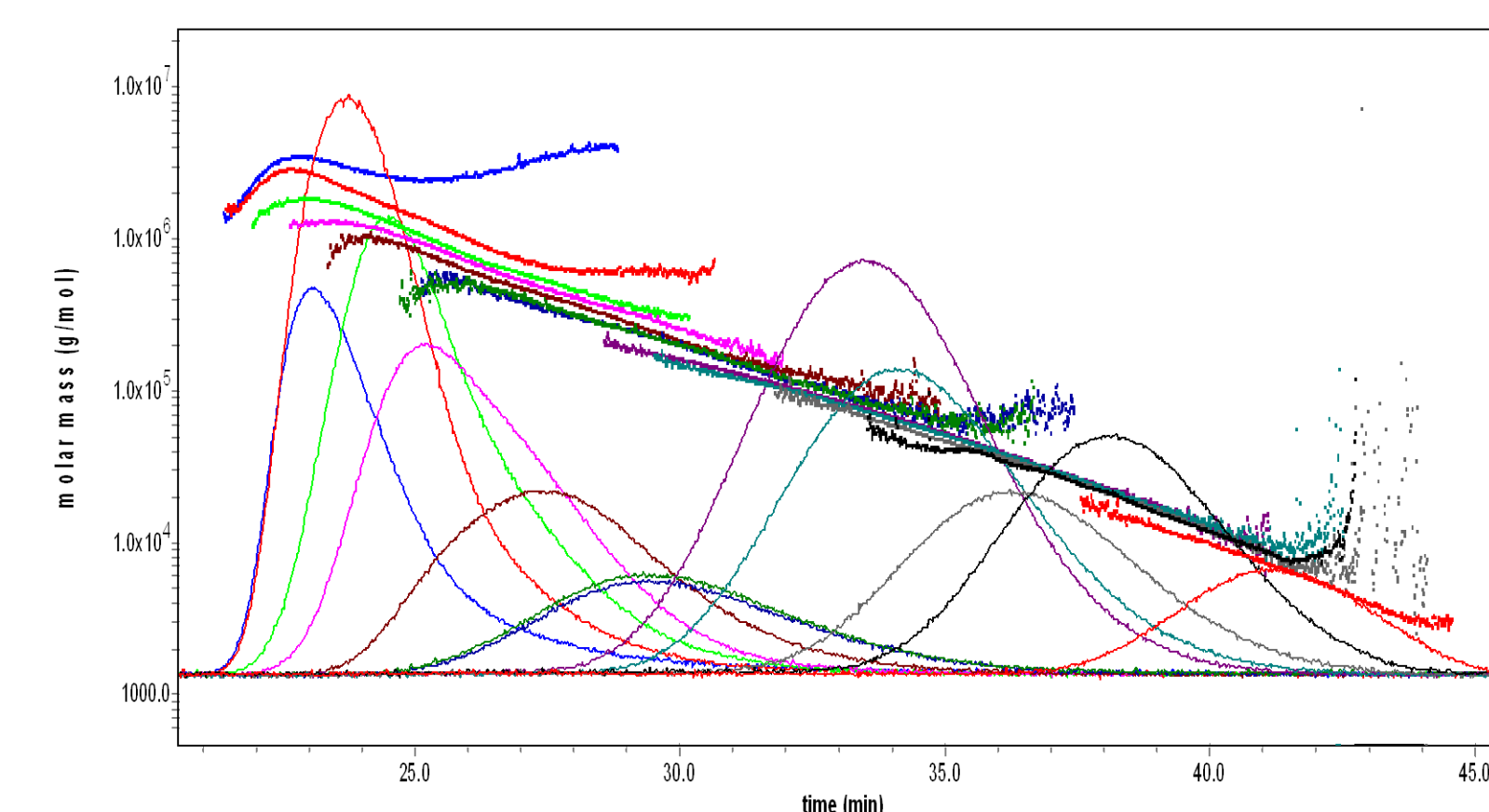
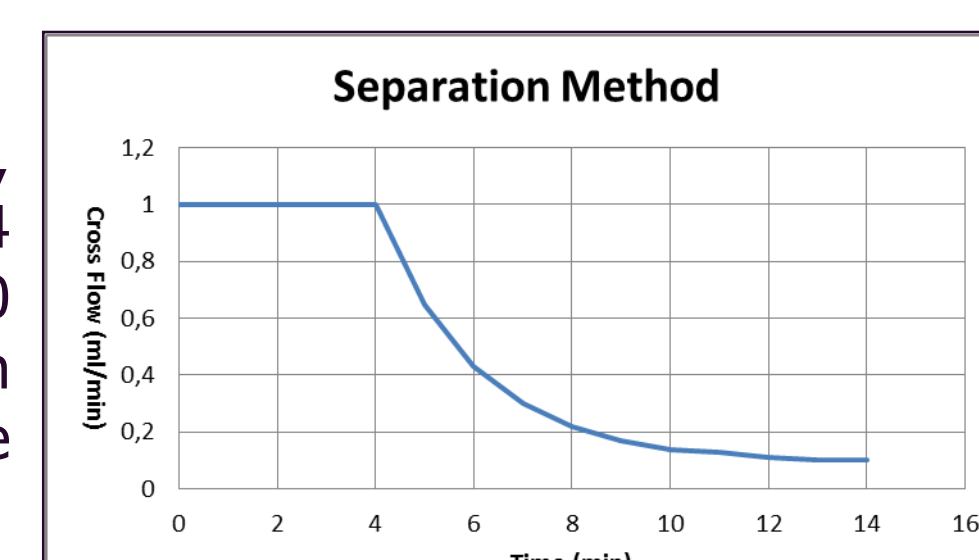


Figure 1: SEC separation of 12 HA samples with molar masses in the range of 7kDa to 2.8 MDa. Light scattering signals and calculated molar masses are shown. Separation of lower molar mass samples is satisfactory, but mass curves of high molar mass samples show poor separation and atypical curve shape („downswing“) at early retention times. Data not shown indicate that the molar mass result can also vary with different SEC column properties like particle size, porosity, etc.

Materials and Methods

- SEC setup: Waters Alliance pump and autosampler, Dawn EOS, Optilab rEX, TSKgel columns G5000PWXL and G6000PWXL, mobile phase 150 mM NaCl, 50 mM NaH₂PO₄, 0.05% NaN₃, pH 7.0
- AF4 setup: Agilent 1200 pump and autosampler, HELEOS II with QELS, Optilab T-rEX, Eclipse 3+ with LC channel, 350 µm spacer
- FI-AF4 setup: Agilent 1200 pump and autosampler, HELEOS II with QELS, Optilab T-rEX and Eclipse AF4 with Frit-Inlet Channel based on LC channel with 350 µm spacer. Injection flow rate was 0.2 ml/min and an exponential decay off the cross-flow was used. The separation method is shown to the right.



Conventional focusing AF4 presents limitations for the characterization of high MW HA

Figure 2 shows separation of a high-MW HA sample using conventional focusing AF4. The experimental conditions were identical, only the sample load was increased. With higher sample load a shift of the 90° light scattering signal is observed as well as an increase of the aggregate fraction of the sample. This effect compromises focusing AF4 for HA characterization.

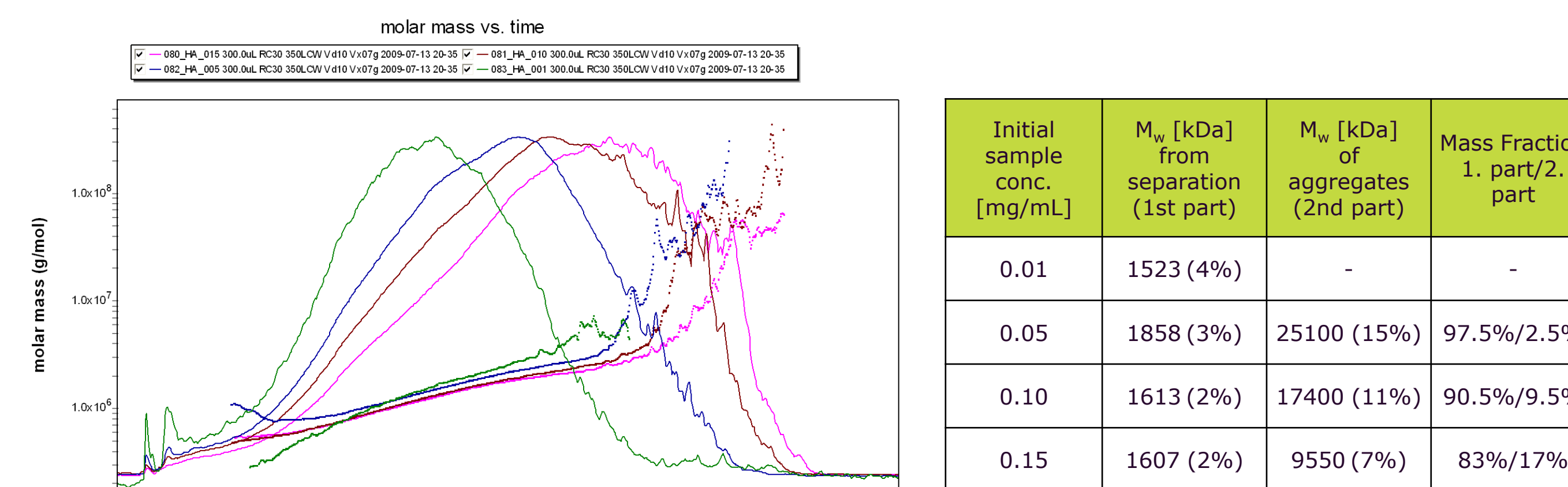


Figure 2: Molar mass vs. elution time of 4 sample runs with different sample load. With increasing sample load the apparent molar mass increases and a strong shift of the LS peak to higher retention time is evident.

Principle of frit inlet AF4

FI-AF4 has a channel design (Fig. 3) modified from AF4 by introducing a small inlet frit at the beginning end of the depletion wall so that sample relaxation can be made hydrodynamically by the use of the compressing action of the frit flow entering through the small inlet frit. In FI-AF4, sample materials are introduced to the channel via the channel inlet at a low speed while a relatively high speed frit flow is entering through the inlet frit. Sample components injected into the channel are pushed toward the accumulation wall by frit flow and they can undergo hydrodynamic relaxation without halting their migration along the channel. Therefore, the separation process can be continuously achieved without stopping the flow. When hydrodynamic relaxation procedure is utilized, injected sample components are expected to achieve relaxation while they continuously migrate along the channel. The continuous fluid motion reduces the chance of sample adhesion to the channel wall.

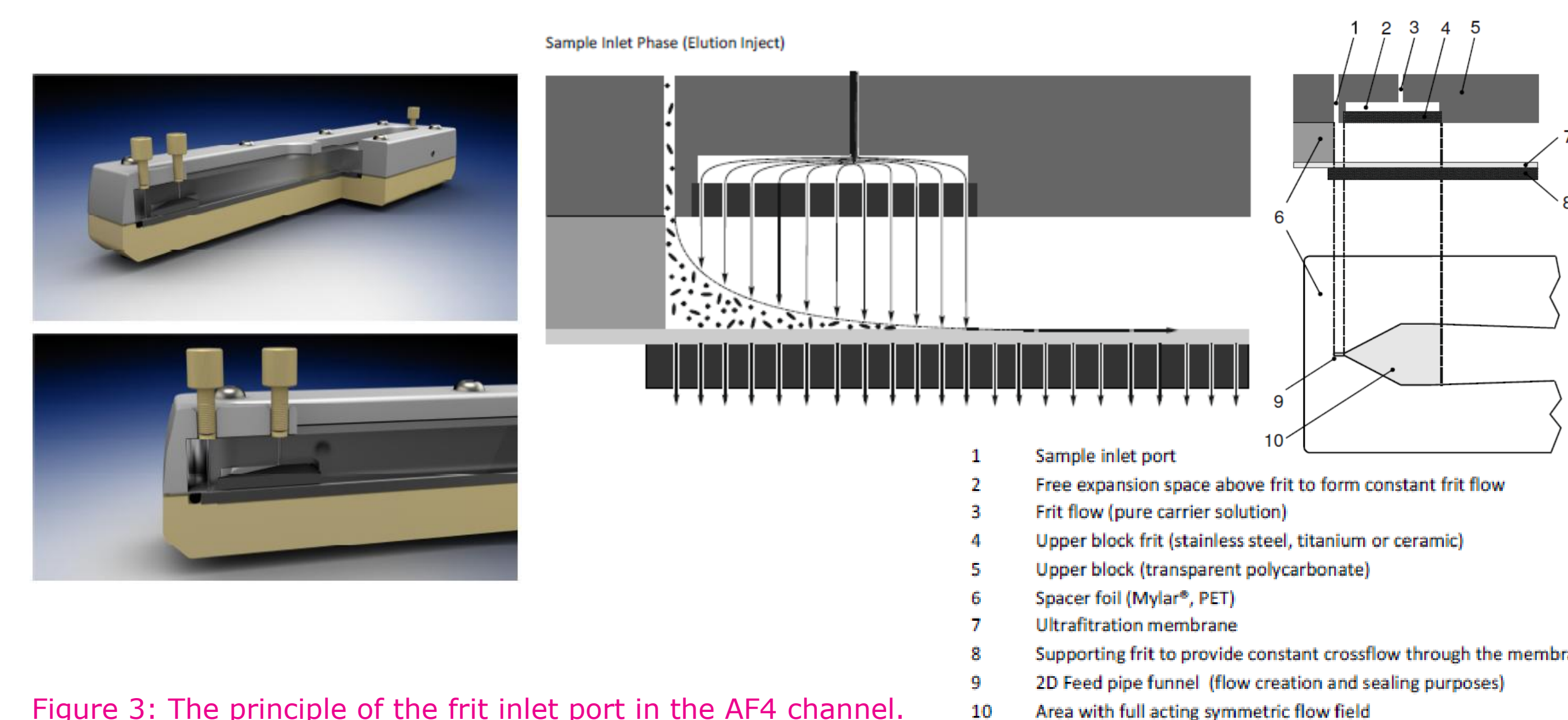


Figure 3: The principle of the frit inlet port in the AF4 channel.

Frit inlet AF4 proved efficient for the separation of high MW HA molecules

The successful use of FI-AF4 for the separation of HA has been shown [1] and we applied this methodology to the separation of the HA samples which had already been analyzed with SEC and AF4. The data presented in Fig. 4 demonstrate that the FI-AF4 mode does not aggregate the sample and leads to an efficient separation.

Still the characterization of HA is challenging. The sample elutes in a broad peak resulting in high dilution and correspondingly low detector signals. This is mainly a problem for the concentration detection, which has to rely on RI, because HA is not UV active. It is a requirement to decay the cross-flow field during elution. In our case an exponential decay was used. This produces a drift in the RI baseline, which needs to be minimized in order to allow a simple linear baseline subtraction. We used a 30 kDa cutoff membrane and a 50 mM NaNO₃ eluent, which minimizes the detector drift effect compared to other buffers (data not shown)

With the Optilab T-rEX it was possible to generate reliable and reproducible concentration signals.

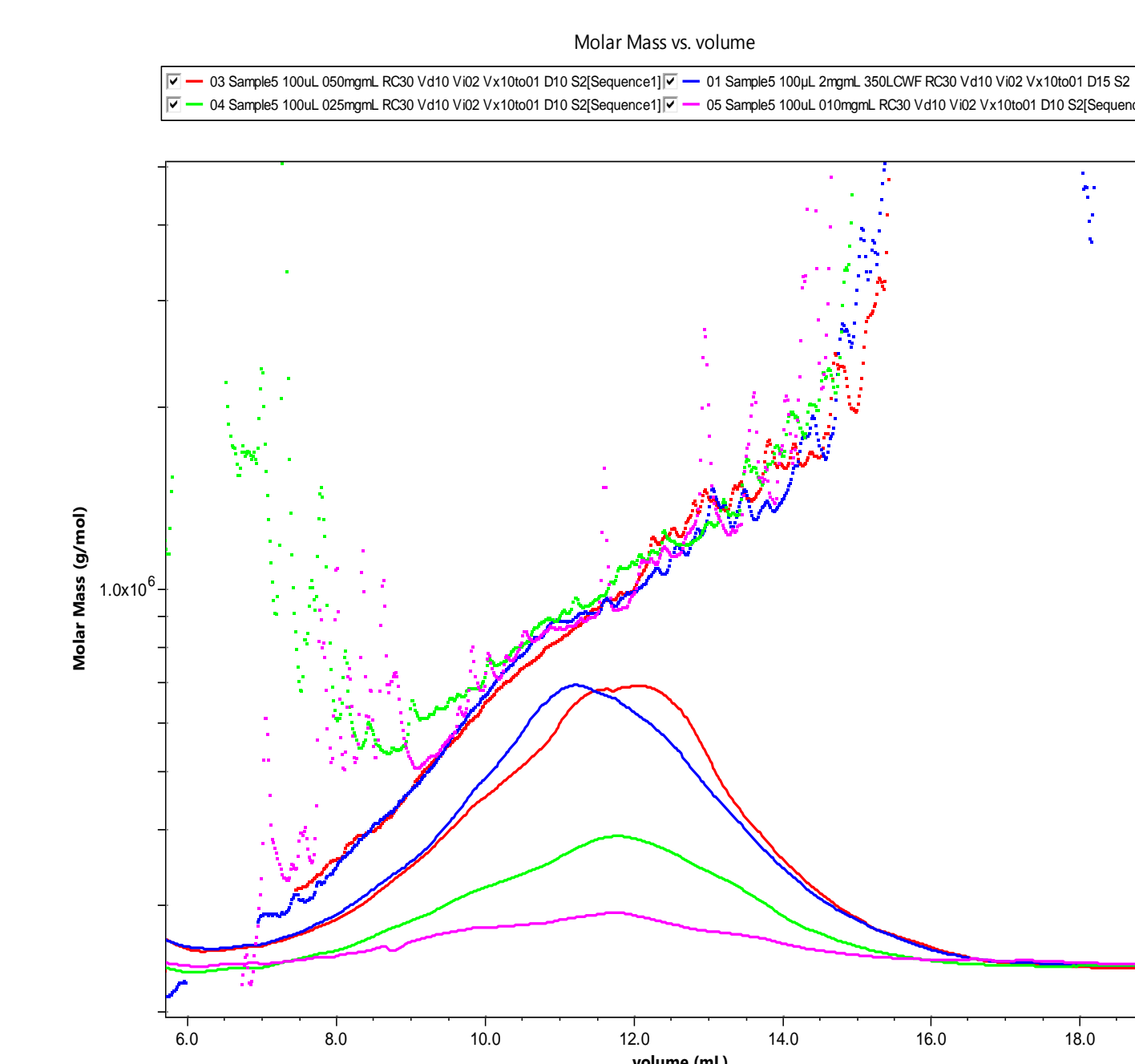


Figure 4: Molar mass vs. elution time of 4 sample runs with different sample load. The overlay shows that the peaks do not shift in retention time with increasing sample load. With optimized buffer (50 mM NaNO₃) and 30 kDa cutoff membrane it was possible to calculate reliable molar mass values.

The table lists details about the injected sample load, molar mass and RMS values. The Rh taken from QELS measurements is also given.

Initial sample conc. [mg/mL]	M _w [kDa] (% STD)	Rz rms [nm] (% STD)	Rh(z) [nm] (% STD)
0.10	871.7 (2.1)	132.7 (1.4)	215.0 (11.1)
0.25	1040.4 (1.7)	127.8 (1.2)	204.0 (5.4)
0.50	1011.8 (3.0)	128.9 (2.0)	187.8 (4.0)
2.00	955.1 (1.7)	126.4 (1.2)	190.9 (3.8)

Conclusions and outlook

Frit-Inlet AF4 is a promising method to separate samples which are prone to aggregate during a focusing step. Therefore it is an important addition to the Flow-FFF methods, because it resolves a critical problem to use AF4 for these types of samples. Resolution is not quite as good compared to conventional focusing AF4, but the experimental procedure is simpler, because a reversal of flow rate direction is not required. This has a positive effect on detector baseline stability. For the first time a commercial instrument for FI-AF4 is introduced, which will help to propagate the use of FI-AF4.

References

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