Hollow Fibre Flow Field Flow Fractionation (HF5) Increases Sensitivity and Efficiency in the Separation of Proteins and Complex Protein Mixtures

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In this contribution we provide insight into the functional principles of flow field flow fractionation (F4) which enables the user to separate macromolecules and particles with high efficiency. The latest development in this field, the hollow fibre technology (HF5), will be introduced. Based on several exemplary measurements we elucidate the advantages of HF5: it provides higher sensitivity compared to SEC and AF4, better separation performance for certain delicate samples (e.g.: antibodies) and increased versatility through the use of a disposable HF5 cartridge, which can be changed within seconds, a new feature that helps to avoid cross contamination and sterility issues.

Introduction

Field-Flow Fractionation (FFF) is a well known family of separation methods for molecules and particles which vary in the physical nature of the force field applied to generate separation [1]. Asymmetric Flow Field-Flow Fractionation (AF4) is the most popular type of FFF. It employs a flat separation channel equipped with a membrane and covers a wide separation range (1 nm - 50 µm).

In this channel two perpendicularly working hydrodynamic forces cause the arrangement of the molecules in the channel space according to their diffusion coefficient. This eventually leads to the fractionated elution of the components depending on their position in the parabolic elution flow stream.

Here we introduce for the first time a special type of FFF, the hollow fibre technique (HF5). In HF5 the solvent is pumped through a porous fibre allowing a part of the flow to penetrate the wall, thus creating a cross flow which is perpendicular to the main solvent flow that has a parabolic profile and is directed to the fibre outlet. The combination of the two forces applied eventually results in the separation of the sample compounds according to their respective diffusion coefficient (i.e. their hydrodynamic radius or molar mass, respectively; see Figure. 1).

Like AF4, HF5 has a wide range of applications. It allows the separation of molecules in solution and particles in the same separation run. This is a key feature when "free" reagents have to be separated from the fraction that is actually "bound" to functional particles [2]. The separation takes place without the use of a stationary phase as in column chromatography. Consequently, there is less danger of sample absorption or physical plugging of the separation channel. Although HF5 has been utilised by only a few research groups, the literature shows promising results for protein, nano particles, and even whole cell fractionation [3-6]. Another advantage of this technique is the low sample dilution due to the small channel volumes (≤ 100 µL) and low detector flow rates. This allows the coupling of HF5 to special mass spectrometry detection methods, e.g. ESI-MS. Certain features of HF5 motivate development of this technique for applications in emerging bioanalytical fields such as protein analysis and proteomics as well as environmental or industrial nano particle analysis [7-10]. The hollow fibres are low-cost materials and allow the construction of a fractionation channel which is disposable. This helps to avoid any problems concerning sterility and sample cross-contamination, since the single use HF5 cartridge can simply be replaced. Moreover, it increases working efficiency and reduces the time needed for analysis in a couple of experimental settings.

Here we present for the first time the new Eclipse DUALTEC F4 system (Wyatt Technology). A new F4 system suitable to be used with both AF4 and HF5 channels. The new system equipped with either a two-port AF4 channel or an HF5 cartridge provides the flexibility to change from AF4 to HF5 mode in one instrumentation and provides an innovative approach to flow rate monitoring and automation of the focusing/relaxation step. It is shown that the new design improves the separation efficiency and repeatability of HF5.

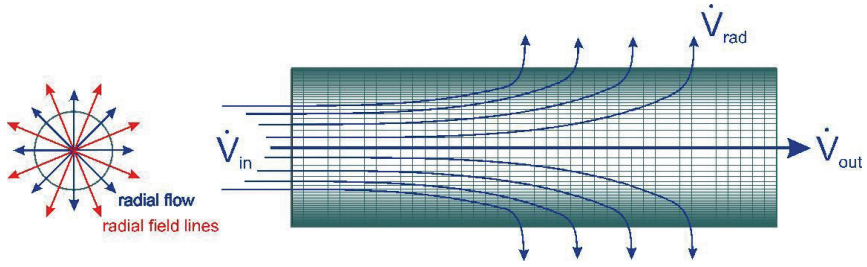

Figure 1: Hollow fibre flow pattern V_{rad}: radial cross flow; V_{in}: pump flow; V_{out}: detector flow

Experimental

HF5 channel design

The HF5 channels are a construction consisting of a tube housing of a hollow fibre, with length of 17 cm, the hollow fibre itself, two cap nuts, two gaskets, and two stainless steel ferrules with an inner chamfer

on one side. The hollow fibre is sealed with the stainless steel ferrule and the gasket. By tightening the cap nut, the ferrule and the gasket compress the hollow fibre to form a seal against the outer volume, ensuring that the flow and the sample are introduced into the fibre. The cartridge is sealed up to 30 bar (435 psi).

Two different fibre types were used. The hollow-fibre material used in the cartridges was polyethersulphone in both cases. One fibre had a nominal molecular weight cutoff of 10 kDa, which corresponds to an average pore size of 5 nm; 0.8 mm ID, and 1.3 mm OD (Fibre type FUS 0181, Microdyn-Nadir, Wiesbaden, Germany). The other fibre had a nominal molecular weight cutoff of 30 kDa, 0.5mm ID, and 1mm OD (Fibre type FUS 0353, Microdyn-Nadir, Wiesbaden, Germany).

Figure 2: A HF5 separation cartridge

Two-port AF4 channel

One of the features of current F4 instrumentation which leads to complications when switching between AF4 and HF5 (or packed column in the same system) is the number of fluid connections of the separation device. AF4 channels usually have three fluid connections in the upper plate [11] (inlet, injection and outlet), whereas HF5 channels have only two, like chromatography columns. Therefore the AF4 channel was newly designed with only two fluid connections in the cover plate for the mobile phase inlet and outlet ports. The sample injection takes place through the mobile phase inlet port. In this novel channel type no spacer is used; instead, the separation space is part of the upper plate. The decision which separation device (cartridge or channel) should be used depends on the type and formulation of the sample. Wyatt's new ISIS software, which was designed to facilitate method development, helps to make this choice.

Instruments, samples, chemicals

In Figure. 3 a schematic view of the new system able to operate either with a two-port AF4 channel or with an HF5 channel is presented. The six-port valve (1) is used to switch between elution and focus mode. During focusing mode, the flow is split into two parts which enter from both fluid connections into the channel device (5). The flow rate in the direction of the inlet (top) port of the channel is measured with a flow meter (4) in real time and regulated by the metering valve (2) to a certain, defined value, through which the flow rate entering at the outlet (bottom) port is automatically determined. The ratio of the two focusing flow rates can be adjusted and this will place the focusing zone at a position given by the same ratio of the distance from the inlet and outlet port. This procedure replaces an empirical balancing of flow rates by injection of a coloured sample (usually dextran blue) It does not require a transparent upper channel plate. In case of an HF5 channel, the ratio can be adjusted for each new fibre installed, even for each focusing process individually. By changing the needle valve position, the focusing band can be shifted and it can be widened. In the latter case this function slightly expands the focusing area. For some special 'sticky' samples (e.g. antibodies) this can make sense, since it reduces concentration in the focusing area and thus minimises the danger of sample aggregation.

During elution the longitudinal flow entering the detectors and the cross flow rate are regulated with a second flow meter with an adjustable metering valve (7).

The HPLC instruments were from Agilent Technologies (Santa Clara, USA) with a 1100 Agilent degasser, a 1100 Agilent isocratic pump, a 1200 Agilent auto sampler, and a 1100 Agilent variable wavelength detector. Samples were solutions of carbonic anhydrase (CAH, 30 kDa), bovine serum albumin (BSA, 66 kDa), apoferritin (APO, 481 kDa), thyroglobulin (THG, 670 kDa) (all analytes from: Sigma, St. Louis, USA) and a monoclonal antibody in the carrier solution. Injected sample amounts were 1µl to 5µl in 1µl steps of a 1mg/mL carbonic anhydrase solution and 5µl for the mixture of proteins (CAH, BSA, APO, THG) with a concentration of 0.1mg/mL each protein, resulting in a total concentration of 0.4mg/mL and 0.3µl of a 0.5mg/mL antibody solution. Carrier solutions were prepared in water purified by an Elix 3 UV Water Purification System (Millipore, Billerica, USA), and filtered through a 0.1µm pore membrane filter. Two different carrier solutions were used: (1) 50 mM NH_4HCO_3 for carbonic anhydrase and protein mixture measurements and (2) 50 mM PBS + 150 mM NaCl (pH 7.2) for the antibody experiment.

Flow conditions of carbonic anhydrase experiments (Hollow fibre (HF) 800µm)

Using the hollow fibre with the inner diameter of 800µm, the fractionation of carbonic anhydrase was performed with a longitudinal flow rate of 0.35ml/min. The focusing step was performed for 4 minutes with a focusing flow rate of 0.85ml/min. During the elution, the cross-flow was maintained constant at 0.85ml/min until the complete elution of sample components.

Flow conditions of protein mixture separations (HF 800µm)

For the separation of the mixture of proteins, the detector flow was set to 0.2ml/min and the cross-flow at 0.85ml/min. During the elution step the cross-flow was maintained constant for 13.5 minutes and then reduced

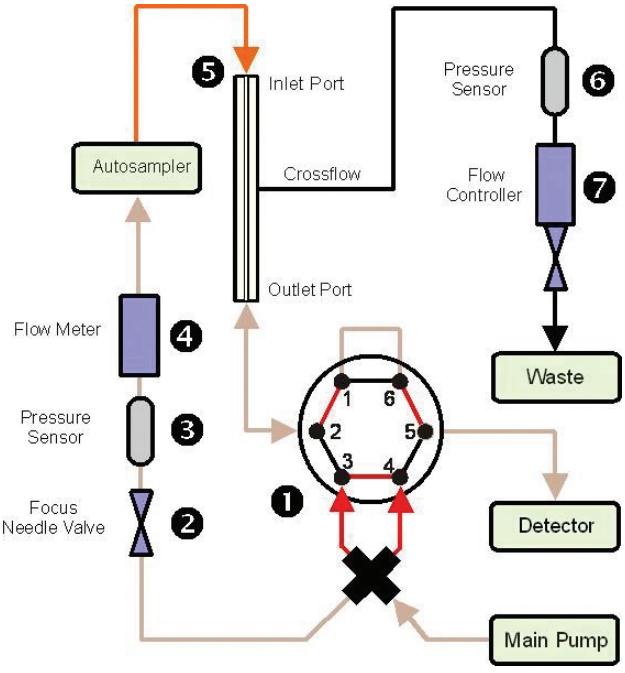

Figure 3: Schematic view of the flow pattern and control elements used in the DUALTEC instrument:
1: six-port valve; 2: metering valve; 3 and 6: pressure sensor; 4: flow meter; 5: separation channel or cartridge; 7: adjustable metering valve

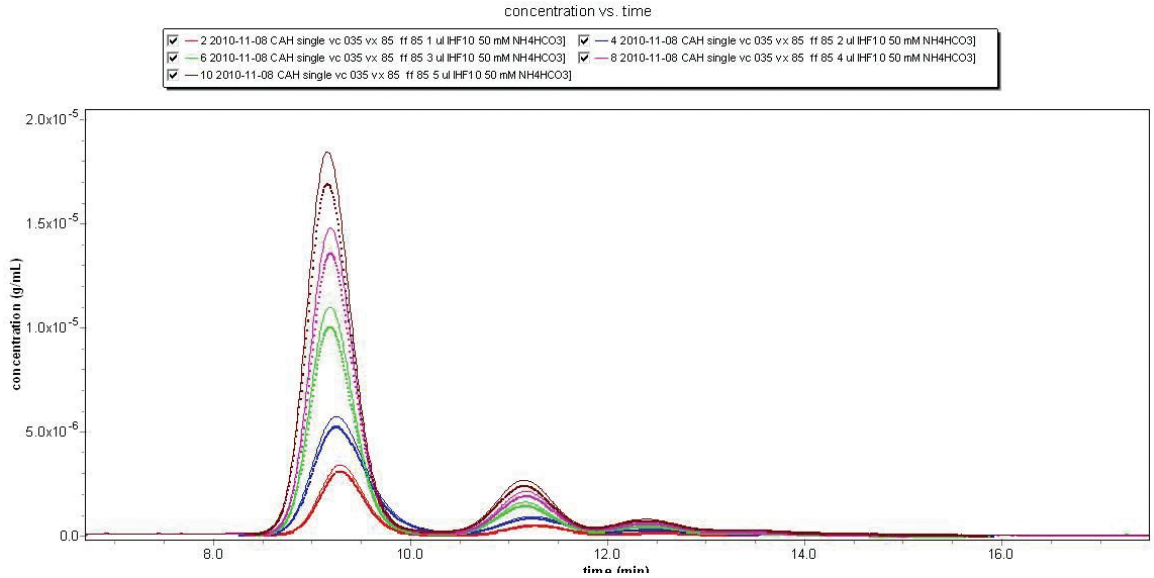

Figure 4: Separation of carbonic anhydrase (1 mg/ml) with hollow fibre (800µm) cartridge, injection amounts increase from 1µg to 5µg, UV signal at 280nm (dotted line: concentrations calculated from UV data)

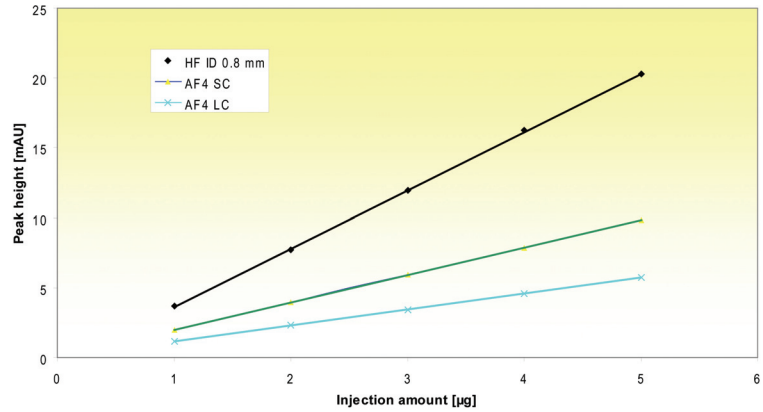

Figure 5: Carbonic anhydrase separation; peak height as a function of injection amount compared to calculated peak heights on AF4 channels SC and LC with 350µm channel height

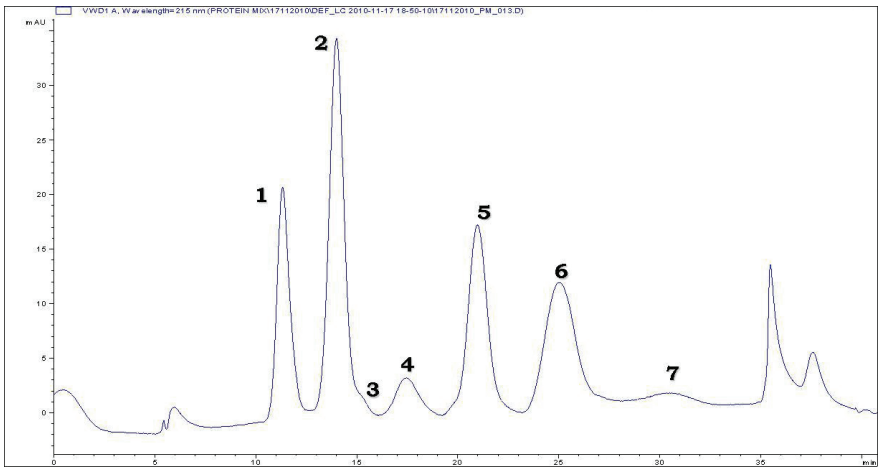

Figure 6: HF5 separation of a protein mixture; UV signal at 215nm

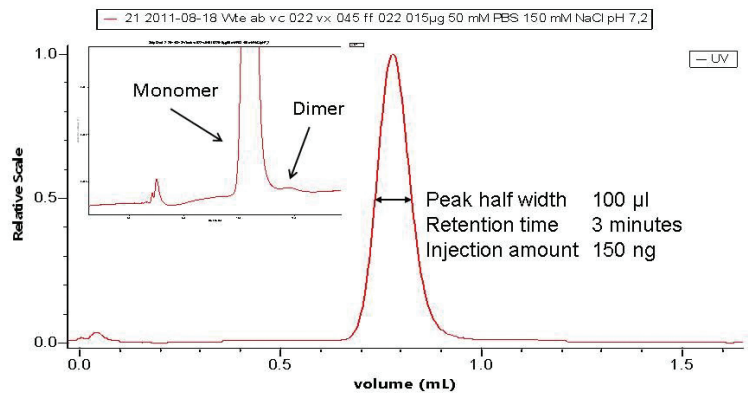

Figure 7: analysis of a monoclonal antibody; UV signal at 210nm

to 0.5ml/min in 0.5 minutes and then maintained constant for 7 minutes, before it was reduced to zero in a linear manner within 9 minutes. The focusing step took 5 minutes at a focusing flow rate of 0.85ml/min.

Flow conditions of antibody experiments (HF 500µm)

Using the hollow fibre with an inner diameter of 500µm, the separation of the antibody samples was performed with a detector flow rate of 0.22ml/min and a constant cross flow of 0.45ml/min during the elution step. The duration of the focusing step was 6 minutes, which was performed with a focussing flow rate of 0.22ml/min.

Results and Discussion

Carbonic Anhydrase separation

This analysis shows excellent reproducibility, high sensitivity and 'baseline' resolution between monomer and dimer of the HF5 separation. Based upon the low sample dilution the sensitivity of detection clearly increases in HF5. Figure 5 shows a comparison of the UV signals as a function of the sample amount applied. Two different channels (short channel (SC) and long channel (LC)) and the HF5 cartridge were used for the analysis of carbonic anhydrase.

Although the channel heights were similar (HF5 radius 400µm, AF4 spacer 350µm), the HF5 separation yields significantly higher peaks (factor 4-6) when compared to data generated using a channel.

To confirm increased resolution and efficiency within a broader molar mass range, a mixture of proteins was run. Figure 6 reports the HF5 fractogram of this separation.

Separation of a complex protein mixture

Peaks represent: 1 - Carbonic anhydrase (monomer), 2 - BSA (monomer) + Carbonic anhydrase (dimer), 3 - Carbonic anhydrase (trimer), 4 - BSA (dimer), 5 - Apoferritin (monomer), 6 - Thyroglobulin (monomer) + Apoferritin (dimer), 7 - Apoferritin (trimer) + Thyroglobulin (dimer)

HF5 yields a good separation of the protein mixture. All components can be clearly separated and identified by comparison with single-injected protein samples. For several protein components, even the detection of dimers was achieved.

mAb analysis

The mAb analysis shows the high sensitivity of the HF5 system yielding narrow peaks with perfect peak symmetry. The insert (top, left hand side) shows that even the extremely small dimer formation (approx. 1% of the protein load) of the antibody can be detected.

Conclusions

The results show that using the new field flow fractionation system design, the gap between HF5 and AF4 is closed with respect to separation efficiency. The new F4 approach proposed here differs in several ways from the systems previously described in the literature:

1. Only one pump is used.

2. The cross flow is regulated with a flow meter device. It is not generated by a pump working in 'unpump' mode.

3. The focus position is determined and regulated in a novel way which avoids possible shifts of the position away from the optimal point. This method also allows dynamic adjustment and shifting of the focussing position.

4. The HF5 cartridge can be manufactured in large quantities. One cartridge is replaced with another in a few seconds by connecting three pieces of tubing with finger tight fittings.

5. HF5 appears to be a very useful addition to the AF4 technology, especially with respect to its high sensitivity when compared to AF4 and SEC standard techniques.

We are confident that the new F4 system offers higher potential to F4 users because of the enhanced HF5 performance and the flexibility to alternatively use AF4 or HF5 channels.

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