Application of microstructured membranes for increasing retention, selectivity and resolution in asymmetrical flow field-flow fractionation

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A B S T R A C T
In the present proof-of-concept study, we demonstrate that retention time, selectivity and resolution can be increased in asymmetrical flow field-flow fractionation (AF4) by introducing microstructured ultrafiltration membranes. Evenly spaced micron-sized grooves, that are placed perpendicular to the channel flow on the accumulation wall of a field-flow fractionation system, cause a decrease in the zone velocity which is stronger for larger solutes. This has been demonstrated in thermal field-flow fractionation, and we prove that this is also the case in AF4. We examine the hypothesis theoretically and experimentally, by both computational and physical experiments. By means of moment analysis, we derive theoretically a set of equations which, under certain conditions, describe the mass transport and relate retention time, selectivity and plate height to the dimensions of the grooves. Physical experiments are carried out using microstructured polyethersulfone membranes fabricated by hot embossing, and the experimental results are compared with computational fluid dynamics experiments.

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1. Introduction

Asymmetrical flow field-flow fractionation (AF4), the most applied subtechnique of the field-flow fractionation (FFF) family, is an established analytical method to separate macromolecules and nanoparticles according to their hydrodynamic size under mild conditions [1–3]. The coupling with various physical and chemical detectors has contributed significantly to its popularity as it can provide valuable information such as molecular weight distribution, size distribution, conformation and chemical composition in a single run [4]. Considering the rapid growth in biotechnology, nanotechnology and polymer engineering, it is evident that AF4 is going to witness a further growth in applications in the coming years. In this regard, it is worthwhile to propose and investigate possible new technical developments that may improve performance.

In this study we investigate the possibility of increasing retention time, selectivity and resolution by using microstructured ultrafiltration (UF) membranes with parallel grooves on their surfaces (Fig. 1). However, considering that AF4 is a very flexible technique where several parameters can be altered to optimize separation, first a justification should be given for the usefulness of such a development.

According to the rigorous FFF theory, the retention time of well-retained (with retention ratio λ < 0.1) components in AF4 is equal to [5],

$$t_R = \frac{w^2}{6D} \ln \left(1 + \frac{V_c}{V_{out}} \frac{B}{B_1}\right)$$

(1)

where w is the channel thickness, \(V_c\) the cross-flow rate, \(V_{out}\) the channel outlet flow rate and \(B\) the fraction of the accumulation area after the focusing point. Therefore, the selectivity of a pair of well-retained solutes equals the ratio of their diffusion coefficients,

$$\alpha = \frac{D_1}{D_2}$$

(2)

and consequently, it cannot be altered by changing the experimental parameters. Resolution can be improved by reducing the plate...
height which, based on the nonequilibrium theory (for $\lambda < 0.1$), is equal to [6],

$$H = \frac{24D^2 \langle \eta_0 \rangle}{u_{cr} w}$$  \hspace{1cm} (3)$$

where $u_{cr}$ is the cross-flow velocity thought the membrane and $\langle \eta_0 \rangle$ is the cross-sectional mean carrier velocity. Hence, a high cross-flow velocity decreases plate height. However, it may lead to adsorption on the membrane and mass overloading for sensitive macromolecules. In addition, high flow rates are hindered by the transmembrane pressure when ultrafiltration (UF) membranes with very low molecular weight cut-off (MWCO) are used to separate small macromolecules.

The solutes can be resolved at lower cross-flow rates by increasing the retention time, since a minimum time is required to achieve separation [7], which could be accomplished by increasing the cross-flow to outlet flow ratio or the spacer thickness [8]. Very high cross-flow to outlet flow ratios are impractical, particularly for UF membranes with low MWCO, and may distort the parabolic flow profile [9]. In addition, the use of a thicker spacer results in higher required focusing times and more dilution with a subsequent decrease in sensitivity [7]. Moreover, a low aspect ratio $b/w$ ($<30$), where $b$ is the channel breadth, may aggravate edge and end effects increasing plate height and reducing recovery [10,11]. Therefore, it could be beneficial to investigate a method that could increase retention and resolution without altering the optimal cross-flow, spacer thickness and cross-flow to outlet flow ratio.

The concept of an accumulation wall with micron-sized grooves in FFF has been introduced in 1978 by Giddings et al. [12] as an attempt to increase retention for small analytes in thermal field-flow fractionation (ThFFF). In addition, grooved surfaces have been incorporated in microfluidic channels for various other applications such as to enable mixing [13] and to separate cells and microparticles [14]. Navier has described that macroscopically the rough surface is equivalent to a smooth surface with partial slip [15–17]. In fact, for this reason, a small slip might exist on the flat membrane of the AF4 channel, as a result to the porosity, but it is negligible for UF membranes [18]. Nanostructured UF membranes have been fabricated by nano-imprinting lithography [19–21], where membranes were hot-embossed, and microstructured polymeric materials have been developed with phase separation [22–24], where a polymer solution is cast over a patterned mold.

The scope of this study is to conduct a proof-of-concept investigation to assess the effect of microstructured membranes on the retention time, selectivity and resolution in AF4. A hot embossing method was chosen for the fabrication of these membranes. We share fundamental theory and experimental findings that complement and expand the previous study with perpendicular grooves in ThFFF [12].

Fig. 1. AF4 with microstructured membranes.

2. Theory

2.1. Transport equations and moment analysis

Here, we describe a simplified model that enables us to derive an analytical solution to the problem of mass migration over grooves in an AF4 channel. In this model the grooves are formed by zero-width ridges with a uniform height $h$ on the membrane surface, perpendicular to the flow direction. Slip flow through the grooves is neglected; the zero-velocity plane for the axial flow ($z$) is taken at the top of the ridges (Fig. 2).

The following simplifications have been made:

1. Molecular diffusion in the axial ($z$-) direction is neglected.
2. The development of the concentration profile in the perpendicular ($x$-) direction is complete before elution is started, by a preceding focusing step in the procedure.
3. Only well-retained compounds are considered (with retention ratio $\lambda < 0.1$). Such compounds are present predominantly close to the accumulation wall, where the linear part of the flow profile prevails and the cross-flow velocity $u_{cr}$ may be considered as being equal to the fluid velocity through the membrane. For well-retained compounds, the mathematics can be simplified since integrals over the height of the channel can be taken from $x = 0$ to infinity instead of to the upper wall position ($x = w$), with good accuracy.
4. There are no interactions between the protein and the membrane.
5. Flow conditions are laminar. This assumption should hold true since the presence of perpendicular grooves, which are small compared to the channel thickness, reduces locally the flow velocity and decreases the Reynolds number [16]. Although eddies may exist in the corners of the grooves, the flow velocity is very low there and the fluid is almost stagnant.

The transport of a compound $i$, with a local concentration $c_i = c_i(x, z, t)$, is given by the simplified general transport equation

$$\frac{\partial c_i}{\partial t} = D_i \frac{\partial^2 c_i}{\partial x^2} + u_{cr} \frac{\partial c_i}{\partial x} - v(x) \frac{\partial c_i}{\partial z}$$  \hspace{1cm} (4)$$

where $D_i$ is the diffusion coefficient of the compound of interest, and $v(x)$ the local axial flow velocity. The molecular diffusion term along the $z$-direction is neglected in the RHS of Eq. (4). The plus sign for the second term of the RHS appears because a positive value is taken for $u_{cr}$, even when the cross flow is in the negative $x$ - direction. The assumption that the analyte has been introduced in the channel as a finite plug leads to the boundary conditions

$$c_i(x, t) \rightarrow 0 \text{ for } z \rightarrow \pm \infty \hspace{1cm} (4a)$$
and the assumption that the walls of the channel are impermeable for the compound to

$$D_i \frac{\partial c_i}{\partial x} + u_\text{cr}c_i = 0 \quad \text{for} \quad x = 0, w$$  \hspace{1cm} (4b)

Two sets of moments are defined. Local moments, that describe the mass distribution of a compound $i$ in a fluid layer at a certain distance $x$ from the membrane, are defined as

$$m_{n,i}(x, t) = \int_{-\infty}^{+\infty} z^n c_i(x, z, t) \, dz$$  \hspace{1cm} (5)

and overall moments, that describe the mass distribution in the axial direction integrated over the height of the channel, as

$$M_{n,i}(t) = \int_0^w m_{n,i}(x, t) \, dx \approx \int_0^\infty m_{n,i}(x, t) \, dx$$  \hspace{1cm} (6)

Moments exist when the integrals converge in Eq. (6), i.e., when it can be assumed that the concentration of a compound $i$ approaches zero fast enough when $z$ goes to plus or minus infinity. This will be the case when the compound was introduced in the channel as a plug or peak of finite width.

When both sides of the general transport Eq. (4) are multiplied with $z^n$ and integrated over $z$ from minus to plus infinity, expressions are obtained for the local moments of $i$

$$\frac{\partial m_{n,i}}{\partial t} = D_i \frac{\partial^2 m_{n,i}}{\partial x^2} + u_\text{cr} \frac{\partial m_{n,i}}{\partial x} + n v(x) m_{n-1,i}$$  \hspace{1cm} (7)

The third term of the RHS in this equation is obtained by partial integration with the assumption that $z^n c_i(x, z, t)$ vanishes for $z \rightarrow \pm \infty$. When a local moment $(n - 1)$ is known, this equation can be used to evaluate the next local moment $(n)$. Integration of Eq. (7) over the height of the channel, considering boundary condition (4b), gives an expression for the overall moment $M_{n,i}$

$$\frac{\partial M_{n,i}}{\partial t} = n \int_0^w v(x) m_{n-1,i} \, dx$$  \hspace{1cm} (8)

### 2.2. The zeroth moment (mass distribution)

Integrating both sides of Eq. (4) over $z$ from minus to plus infinity gives an expression for the local zeroth moment of $i$,

$$\frac{\partial m_{0,i}}{\partial t} = D_i \frac{\partial^2 m_{0,i}}{\partial x^2} + u_\text{cr} \frac{\partial m_{0,i}}{\partial x}$$  \hspace{1cm} (9)

Under the assumption that focusing was complete, and a steady state was reached before the experiment was started, both sides of Eq. (9) must be zero, and the mass distribution over the height of the channel can be found as

$$m_{0,i} = m_{0,i}^* \exp \left( - \frac{u_\text{cr}}{D_i} x \right)$$  \hspace{1cm} (10)

where $m_{0,i}^*$ is the zeroth moment on the membrane surface (with $x = 0$). The exp. concentration profile extends out from the upper wall $(x = w)$ here. Eq. (10) describes the well-known exponential concentration profile on the accumulation wall in FFF, with a characteristic layer thickness ($\ell$) equal to $D_i/u_\text{cr}$. When the concentration of the analyte is scaled so as

$$m_{0,i}^* = \frac{u_\text{cr}}{D_i}$$  \hspace{1cm} (11)

the overall zeroth moment $M_{0,i}$ becomes 1, and the higher overall moments are automatically normalized.

### 2.3. The first moment (mean retention time)

The model for the grooved surface used here (Fig. 2), with a stagnant layer of fluid determined by the groove height $h$, and approximately linearly increasing channel flow rate from the slip plane at the top of the ridges, gives for the local axial flow velocity

$$v(x) = 0 \quad \text{for} \quad 0 \leq x \leq h$$  \hspace{1cm} (12a)

$$v(x) = \frac{6(x - h)}{w} \quad \text{for} \quad x \geq h$$  \hspace{1cm} (12b)

When Eqs. (10)–(12) are substituted into Eq. (8),

$$\frac{\partial M_{1,i}}{\partial t} = \int_0^w \frac{6(x - h)}{w} \langle v \rangle_{D_i} \exp \left( - \frac{u_\text{cr} h}{D_i} \right) \, dx$$  \hspace{1cm} (13)

the axial velocity $v_1$ of the compound is obtained (with for simplicity integration to infinity instead of to $x = w$),

$$v_1 = \frac{\partial M_{1,i}}{\partial t} = \frac{6D_i}{u_\text{cr} w} \langle v \rangle_{D_i} \exp \left( - \frac{u_\text{cr} h}{D_i} \right)$$  \hspace{1cm} (14)

Eq. (14) with $h = 0$ gives the well-known expression for the zone velocity over a flat membrane. With a grooved membrane, the velocity decreases exponentially with the ratio of the ridge height over the characteristic layer thickness. For the retention time the opposite can be written

$$t_{R,i} = t_{R,i}^0 \exp \left( - \frac{u_\text{cr} h}{D_i^0 \ell} \right)$$  \hspace{1cm} (15)

where $t_{R,i}^0$ is the retention time with a flat membrane, under otherwise the same conditions. The retention time increases more strongly by the presence of the grooves for compounds with a small layer thickness, i.e., for more strongly retained compounds.

In the separation of two components, the selectivity $\alpha$ is increased with increasing ridge height and it can be written as

$$\alpha = \frac{t_{R,2}}{t_{R,1}} = \frac{D_{i2}}{D_{i1}} \exp \left( \frac{1}{D_{i2}} \left( \frac{1}{D_{i1}} - \frac{1}{D_{i2}} \right) \right) = \alpha^R \exp \left( \frac{h}{\ell} (\alpha^R - 1) \right)$$  \hspace{1cm} (16)

where $\alpha^R$ is the selectivity with a flat membrane, and $\ell$, the characteristic layer thickness of the first, least retained compound. In Fig. 3a, the calculated effect of the (relative) height of the grooves on the retention times and the selectivity is shown for two compounds with diffusion coefficients that differ by a factor of $\sqrt{2}$.

### 2.4. The second moment (peak variance)

To evaluate the influence of the grooved surface on peak broadening, first an expression for the development of the local first moments has to be derived. In this, we follow the approach taken by Taylor and Aris in their treatment of peak broadening in cylindrical channels, and in early work of Giddings on dispersion in FFF [25]. They found solutions for the general transport Eq. (4) in the form of a sum of transient functions and a stationary function. The transient functions describe the concentration changes in time and space directly after the start of the ‘elution’ and they depend on the initial conditions. It was shown that these transient functions die out rapidly, and that a stationary situation develops in which the local centers of gravity at different distances from the wall are situated in a steady profile around the overall (mean) center of gravity of the transported plug of the compound of interest. Here, a solution is sought for Eq. (4) describing only the stationary situation, i.e., a solution that oblige

$$\frac{1}{m_{0,i}(x)} \frac{\partial m_{1,i}(x)}{\partial t} = \frac{\partial M_{1,i}}{\partial t} = v_i \quad \text{for all} \quad x$$  \hspace{1cm} (17)
increase normalized mB mA A

Fig. 4

The increase in selectivity and retention time for two solutes with diffusion coefficients that differ by a factor of \(2\) (e.g., monomer and dimer); retention times here are normalized with the retention time of the smaller solute for a flat membrane \(\frac{\Delta m}{\Delta t}\)

\(\frac{\partial \Delta M_{2,i}}{\partial t} = \frac{\partial M_{2,i}}{\partial t} - 2M_{1,i}(t)\frac{\partial M_{1,i}}{\partial t}\) \hfill (20)

and finally, the plate height \(H\) can be obtained as

\(H = \frac{\partial \Delta M_{2,i}}{\partial M_{1,i}/\partial t}\) \hfill (21)

The final result for \(H\) is

\(H = \frac{24D_i^2(w)}{u_i^2 w} \left\{ \frac{5}{2} \exp \left( \frac{u_i}{D_i} - \frac{u_c}{D_i} \right) - \frac{3}{2} \frac{u_c}{D_i} \exp \left( \frac{u_c}{D_i} \right) \right\} \) \hfill (22)

For a flat membrane, with \(h = 0\), the second and third factors in the RHS of Eq. (22) are equal to 1, and the well-known expression for \(H(H^*)\) is obtained (Eq. (3)).

In Fig. 3b the increase of the plate height with the relative ridge height is shown, and in Fig. 3c the increase in resolution of two solutes with ratio of diffusion coefficients \(\sqrt{2}\) is shown. We observe that for groove height \(h = 1.5\)kDa, there is a two-fold increase in resolution and a four-fold increase in the retention time of the less retained component. For comparison, the same increase in resolution could be achieved (without altering the cross flow) by a two-fold increase of the spacer thickness or approximately ten-fold increase of the cross-flow to outlet flow ratio.

3. Materials and methods

3.1. Samples and carrier eluent

Bovine serum albumin (BSA), \(\gamma\)-globulin, apoferritin, thyroglobulin and hemoglobin were purchased by Sigma–Aldrich (MO, USA).

PBS 0.15 M (20 mM due to sodium phosphate salts) with a pH of 7.2 was used as a carrier eluent for the AF4 experiments and as a diluent for the proteins. All protein samples were prepared at a concentration of 1 mg/mL.

3.2. Fabrication and characterization of the microstructured (MS) membranes

Two silicon mold designs with parallel grooves were used for preparation of the microstructured membranes. Mold I (LioniX BV, The Netherlands) had a patterned area of diameter 15.1 cm with grooves of cavity width \(c = 50\) m, ridge width \(r = 50\) m, ridge height \(h = 12\) m whereas Mold II (MESA + cleanroom, University of Twente, The Netherlands) had a patterned area of diameter 6.8 cm with grooves of \(c = 30\) m, \(r = 20\) m and \(h = 25\) m. Polyethersulfone (PES) membranes with 10 kDa and 30 kDa molecular weight cut-off (MWCO) (Sartorius, Germany) were used for the membrane patterning without any pretreatment.

Microstructured (MS) membranes were prepared via hot embossing which was performed with an imprinter (Obducat, Sweden) in MESA + cleanroom (University of Twente). The embossing temperature, pressure and time were 120 °C, 40 bar and 180 s, respectively and demolding occurred at 40 °C [20]. Surface and cross-section images of the microstructured membranes were taken by scanning electron microscopy (SEM) equipment, XL30 ESEM-FEG (Philips, The Netherlands) or JEOL JSM-6010 LA (JEOL, Japan). MS membrane I (Fig. 4a) was fabricated by hot-embossing a PES 10 kDa membrane with the Mold I, and MS membrane II (Fig. 4b) by hot embossing a PES 30 kDa membrane with the Mold II. Membrane samples were washed, dried, broken in liquid nitrogen for cross section images and gold-sputtered for SEM imaging.
Clean water flux ($J_w$) values of the membranes were measured with dead-end Amicon Stirred Cell (Model 8050, Merck Millipore, MA, USA) and ultrapure water (MilliQ system, Merck Millipore). Measurements were performed at four different transmembrane pressures ($\Delta P$) in the range of 0.5–2 bar, after removing of the membrane preservatives by immersing in water and after pre-compression at 2 bar. The weight of permeated water versus time was measured and the clean water flux ($J_w$ in L/m²/h) was calculated for each pressure considering the effective membrane surface area, which was $13.4 \text{ cm}^2$ (The area is assumed as constant after preparation of a microstructured surface). The clean water permeance (CWP, in L/m²/h/bar) of the membrane was determined from the slope of $J_w$ versus $\Delta P$ relationship.

### 3.3. AF4 experiments

The AF4 system was an Eclipse DualTec system (Wyatt Technology Europe, Germany) connected to an Agilent HPLC 1200 system (Agilent Technologies, Germany) that consisted of a degasser, an isocratic pump, a UV detector and an autosampler equipped with a thermostat. The temperature of the autosampler was set at 5 °C. Two AF4 trapezoidal channels were used, designated as Channel I and II, one for each membrane/mold size (Fig. 4). The MS membranes were cut with the grooves perpendicular and in the shape of the porous frit with surgical scissors.

Channel I was a commercial AF4 channel (Wyatt Technology Europe) which was used with the larger patterned membrane (d = 15.1 cm). It had tip-to-tip length 13.3 cm and accumulation area $15.6 \text{ cm}^2$ (Fig. 4a). The nominal spacer thickness was 250 or 350 μm. The focus-flow was 1.5 mL/min for 3 min and the focusing point was set at 18% of the channel length. The injected volume was 10 μL (10 μg injected mass) and the UV detection was at 280 nm.

Channel II was a miniaturized channel created to test the smaller patterned membrane (d = 6.8 cm). It had tip-to-tip length 6.3 cm and accumulation area $7.24 \text{ cm}^2$ (Fig. 4b). It was created using a commercial channel modifying its upper inlay and spacer. In the upper inlay two internal threads were milled to connect the tubing fittings for the inlet and outlet. The spacer was fabricated cutting Mylar A4 sheets of nominal thickness 250 and 350 μm. The focus-flow was 0.8 mL/min applied for 3 min and the focusing point was set at 18%. The injected volume was 5 μL (5 μg injected mass) and UV detection was at 220 nm.

### 3.4. Computational fluid dynamics (CFD)

A finite element solver, COMSOL Multiphysics 5.2 (COMSOL Inc., MA, USA), was used to model the AF4 channel and simulate the protein migration over the flat and the patterned membrane. To reduce the model into two dimensions for lower computational cost, a symmetrical channel was modelled instead of an asymmetrical. For this purpose, a simple rectangular domain was created, with a flat or grooved bottom boundary. A mesh of free triangular elements was created with very fine elements (<1 μm) in the proximity to the bottom boundary to simulate protein migration with high accuracy.

To describe the flow, laminar flow of an incompressible fluid was used and the boundary conditions (inlets, outlets) were set to define channel flow and cross-flow velocities (it was verified later from the results that the assumption of the laminar flow was valid by the cell Reynolds number). The cross-flow velocity was distributed homogeneously along the bottom boundary (membrane). The option “transport of dilute species” (including convection and diffusion) was used to simulate protein monomer and dimer. The study of the flow profile was solved as a steady state problem and the output (velocity field) was used to solve the time dependent problem of the protein migration with a BDF (Backwards Differential Formula) solver. The relative and the absolute tolerances were set at $10^{-4}$. The initial and the maximum time steps were set 0.001 s and 0.5 s, respectively.

### 4. Results and discussion

#### 4.1. Characterization of the microstructured membranes

The microstructured membranes, designated as MS membrane I and II had similar ridge height, $h \sim 12 \mu m$, and different peri-
Table 1
Protein recovery in AF4 before and after hot embossing of the UF membranes. AF4 conditions: $V_i = V_{out} = 1 \text{mL/min}$.

| Recovery (%) ± s.d. | BSA (66.5 kDa) | γ-Globulin (150 kDa) | Apoferritin (443 kDa) | Thyroglobulin (669 kDa) |
|---------------------|----------------|---------------------|----------------------|------------------------|
| Flat membrane (10 kDa) | 89 ± 2 | 86 ± 2 | 87 ± 3 | 78 ± 4 |
| MS membrane I | 22 ± 4 | 35 ± 5 | 86 ± 1 | 76 ± 3 |
| Flat membrane (30 kDa) | 20 ± 3 | 60 ± 5 | 82 ± 4 | 71 ± 2 |
| MS membrane II | 9 ± 1 | 11 ± 3 | 84 ± 2 | 72 ± 3 |

Protein recovery after hot embossing should indicate an increase in the actual MWCO rather than protein adsorption since the PES membranes used in this study are hydrophilic with low fouling properties for protein solutions. This was confirmed by injecting and focusing for several minutes a high volume (100 μL) of a concentrated solution (30 mg/mL) of hemoglobin (~65 kDa) which has a red color. It was observed that the sample was focused as a narrow band with a flat membrane while it was passing through the cross-flow with an MS membrane. When the membrane was removed and visually inspected, it was not stained which would indicate adsorption.

The aforementioned results (increase in MWCO and decrease in CWP) seem contradicting since lower CWP is often correlated with a decrease in the size or number of the pores of the selective (patterned) side. A possible explanation is that the CWP decreases because of the membrane compaction (particularly in the area of the grooves’ valleys which experience the highest stress during hot embossing). In addition, the increase in the actual MWCO might be related to an increase of the pore size of the grooves’ ridges because of the membrane deformation or to other local defects that occur during imprinting/demolding which are, however, small enough to affect only the recovery of the smaller proteins.

In contrast with our observations, Maruf et al. [20] showed that hot embossing could lead to similar CWP and lower MWCO for another PES membrane and a mold patterned with smaller grooves (in the sub-micron range). Perhaps the pore deformation there was minimal because of the smaller size of the grooves. However, the effect of the membrane compaction on the CWP and the difference in the stress distribution on the valleys and on the ridges during hot embossing have been discussed in these studies [21]. Overall our results indicate that hot-embossing needs to be optimized to avoid changes of the MWCO since the concept would be beneficial particularly for low molecular weight analytes, and in general UF membranes with high solvent permeability are preferred in AF4.

Using BSA as the calibrant with known diffusion coefficient (6.21 × 10⁻¹¹ m²/s [26]), the actual channel thickness for the Channel I and the Channel II with a flat membrane was estimated 305 ± 6 μm and 294 ± 8 μm respectively, and the diffusion coefficient of apoferritin was estimated 3.38 × 10⁻¹¹ m²/s from Eq. (1). These values were used in the simulations. However, MS membranes are already compressed due to hot embossing, and hence any additional compression caused by the spacer is expected to be small. This would result in larger actual channel thickness and consequently in longer retention times. The difference in compression between the flat and the MS membranes was evident by visual inspection when the membranes were removed from the channel and inspected. Unfortunately, the method with a protein of known diffusivity cannot be applied for the MS membranes as the retention time increases by the presence of the grooves for well-retained compounds. However, in order to assess correctly the effect of grooves, the actual channel thickness of the MS membranes needs to be measured and we attempted this by other means.

First, the membrane compressibility was estimated from the difference in the thickness of the compressed and non-compressed part of the membranes, measured by SEM and a micrometer screw gauge when they were removed from the channel. The compression that occurred with a flat and a MS membrane was ~50 μm and ~20 μm respectively. This corresponds to 11% larger channel thickness with the MS membranes. However, these methods have low precision since they measure only a very small part of the total channel area when the membranes are dry. Second, we applied the rapid breakthrough method [27] in the fractograms obtained for the recovery experiments with injection and elution of thyroglobulin (without the application of focus or cross-flow). The void volume was measured 13% larger with the MS membrane which corresponds to a 13% thicker channel. This result is in close agreement with the first method. However, both methods do not use cross-flow which might slightly affect the membrane compression and/or swelling.

4.2. AF4 experiments

Apoferritin and thyroglobulin were chosen as the model proteins to assess the effect of the grooves on retention time, selectivity and plate height, since they exhibited high recoveries with the patterned membranes (Table 1). In Fig. 5 the fractograms of apoferritin, obtained using flat and MS membranes, are overlaid after subtraction of the time that was required for the focusing step. For both
Fig. 5. Comparison of flat and MS membranes analyzed with flow rates $V_c = V_{out} = 1.0 \text{ mL/min}$ for a) Channel I and MS membrane I and b) Channel II and MS membrane II.

Fig. 6. CFD model for the Channel II/MS membrane II system: a) Mesh of the model in the beginning of the channel, b) velocity profile over the grooves, c) concentration profile of apoferritin over the grooves and d) derived concentration at the outlet (right boundary) for every time point for the monomer and dimer.
channels/MS membrane systems and the same spacer thickness of 350 μm (Fig. 5 left-hand figures), there is a considerable increase in retention time, selectivity and resolution between monomer and dimer. Although there is more peak broadening with the presence of the grooves, resolution is higher because of the higher selectivity (as expected by the theory, Fig. 3). Consequently, the same resolution could be achieved with the MS membranes and applying a lower cross-flow rate, or alternatively using a thinner spacer (Fig. 5 right-hand figures).

Therefore, the MS membranes could be beneficial as the same retention and resolution could be achieved with lower cross-flow rates without the need to increase spacer thickness or to use impractical cross-flow to outlet flow ratios. In practice that would be particularly useful for relatively small solutes (such as BSA or even smaller) since larger solutes can be analyzed with optimal spacer thickness and flow rates, and therefore there is a need to fabricate MS membranes with lower MWCO. The challenges and the procedures to optimize AF4 methods for small solutes with a 300 Da MWCO membrane have been reported [28]. Smaller macro-molecules are typically analyzed by size exclusion chromatography (SEC) where they exhibit very good resolution, but in some cases, SEC is not suitable, for instance, when there is strong non-specific adsorption in the chromatographic support and when large macro-molecules co-exist in the sample that need to be analyzed. In the last case, a cross-flow program with exponential decay should be used as the grooves would cause very strong retention for the large components.

A series of experiments were carried out using different cross-flow rates while retaining the ratio (Vc/Vout = 1); the results are displayed in Table 2. The plate height of the monomer was estimated from the width at half peak height. A number of conclusions may be drawn from these experimental results. Although there is a large departure of the engineered grooves from the theoretical model (i.e., no slip, infinitesimal ridge and rectangular shape), the underlying conclusions were found similar.

First, from Table 2, it can be seen that for higher Vc and same Vc/Vout, the retention time of the monomer and the selectivity between monomer and dimer were similar for the flat membranes as expected by the theory (Eqs. (1) and (2)) but increased for the MS membranes. This is in line with the theoretical equations derived by moment analysis (Eqs. (15) and (16)). Secondly, for the same experimental conditions, the increase in selectivity was higher for thyroglobulin (lower ɛ) and it was independent of the spacer thickness, as predicted by the theory. Lastly, the increase in retention time and selectivity for the same cross-flow velocity was higher for the MS membrane II, probably due to the smaller slip because of the smaller periodicity of the grooves.

It is however important to note that part of the increase in retention time is a result of the larger actual channel thickness with the MS membranes. As it was mentioned above, the channel thickness was estimated ~12% larger with MS membranes, which corresponds to ~25% longer retention times caused by the effect of the membrane compression, as the retention time is proportional to w2 (Eq. (1)). Even so, in the experimental results (Table 2) we observe a much higher increase in the retention times, namely from 67% (for Channel I and a cross-flow rate of 0.8 mL/min) to 180% (for Channel II and a cross-flow rate of 1.0 mL/min) for the monomer of apoferritin, which indicates that the effect of the grooves has the largest contribution to the increase in the retention time. Moreover, overloading was investigated by injecting different sample mass, namely 2 μg, 10 μg, and 20 μg, in the Channel I/MS membrane II system; no overloading effect was observed as retention time, plate height and selectivity were practically the same for every examined injected mass.

4.3. Computational fluid dynamics

For the CFD experiments, the miniaturized channel (Channel II in Fig. 4) was modelled and the migration of the apoferritin monomer and dimer was simulated. The diffusion coefficients for the monomer and for the dimer of apoferritin were taken from the AF4 experiments (where the ratio of the diffusion coefficients, and therefore the selectivity was 1.34). The model was verified by reducing significantly the size of the mesh elements, the time

| Table 2 |
| Comparison of flat and MS membranes for both channels with respect to the plate height of the monomer H, the retention time of the monomer tR, and the selectivity ɛ between the monomer and the dimer. The error bars are given at 1σ level and reflect the membrane-to-membrane reproducibility. |

| Channel I | Apoferritin | Thyroglobulin |
|-----------|-------------|---------------|
| Vc / Vout (mL/min) | H (mm) | tR (min) | ɛ | H (mm) | tR (min) | ɛ |
| Flat membrane, w = 350 μm | 0.8 / 0.8 | 0.88 ± 0.02 | 4.66 ± 0.17 | 1.35 ± 0.02 | 0.69 ± 0.01 | 6.32 ± 0.30 | 1.35 ± 0.01 |
| 1.0 / 1.0 | 0.66 ± 0.02 | 4.62 ± 0.25 | 1.36 ± 0.01 | 0.54 ± 0.02 | 6.26 ± 0.33 | 1.36 ± 0.00 |
| 1.5 / 1.5 | 0.43 ± 0.01 | 4.51 ± 0.16 | 1.37 ± 0.02 | 0.42 ± 0.01 | 6.33 ± 0.31 | 1.36 ± 0.02 |
| MS membrane I, w = 350 μm | 0.8 / 0.8 | 1.03 ± 0.01 | 7.78 ± 0.04 | 1.42 ± 0.01 | 0.87 ± 0.02 | 11.15 ± 0.24 | 1.46 ± 0.01 |
| 1.0 / 1.0 | 0.78 ± 0.03 | 8.09 ± 0.08 | 1.45 ± 0.00 | 0.69 ± 0.01 | 11.45 ± 0.18 | 1.51 ± 0.01 |
| 1.5 / 1.5 | 0.65 ± 0.04 | 8.25 ± 0.23 | 1.49 ± 0.01 | – | – | – |
| MS membrane I, w = 250 μm | 0.8 / 0.8 | 1.27 ± 0.02 | 4.59 ± 0.04 | 1.44 ± 0.01 | 0.91 ± 0.04 | 6.57 ± 0.15 | 1.45 ± 0.02 |
| 1.0 / 1.0 | 0.93 ± 0.05 | 4.92 ± 0.37 | 1.46 ± 0.02 | 0.79 ± 0.02 | 6.63 ± 0.10 | 1.48 ± 0.01 |
| 1.5 / 1.5 | 0.64 ± 0.00 | 5.00 ± 0.04 | 1.51 ± 0.01 | 0.54 ± 0.01 | 7.39 ± 0.04 | 1.53 ± 0.02 |

| Channel II | Apoferritin | Thyroglobulin |
|-----------|-------------|---------------|
| Vc / Vout (mL/min) | H (mm) | tR (min) | ɛ | H (mm) | tR (min) | ɛ |
| Flat membrane, w = 350 μm | 0.5 / 0.5 | 0.27 ± 0.01 | 4.25 ± 0.23 | 1.34 ± 0.01 | 0.21 ± 0.00 | 5.90 ± 0.48 | 1.33 ± 0.00 |
| 0.8 / 0.8 | 0.16 ± 0.00 | 4.26 ± 0.32 | 1.35 ± 0.02 | 0.16 ± 0.02 | 5.92 ± 0.46 | 1.33 ± 0.01 |
| 1.0 / 1.0 | 0.13 ± 0.00 | 4.33 ± 0.17 | 1.35 ± 0.01 | 0.15 ± 0.00 | 5.89 ± 0.47 | 1.34 ± 0.01 |
| 0.5 / 0.5 | 0.43 ± 0.01 | 8.88 ± 0.14 | 1.49 ± 0.01 | 0.39 ± 0.02 | 13.16 ± 0.13 | 1.50 ± 0.01 |
| MS membrane II, w = 350 μm | 0.8 / 0.8 | 0.33 ± 0.01 | 9.90 ± 0.08 | 1.55 ± 0.01 | 0.36 ± 0.01 | 14.56 ± 0.09 | 1.57 ± 0.02 |
| 1.0 / 1.0 | 0.29 ± 0.00 | 12.22 ± 0.05 | 1.59 ± 0.01 | – | – | – |
| 0.5 / 0.5 | 0.53 ± 0.01 | 4.61 ± 0.03 | 1.48 ± 0.00 | 0.47 ± 0.00 | 6.80 ± 0.14 | 1.53 ± 0.02 |
| MS membrane II, w = 250 μm | 0.8 / 0.8 | 0.34 ± 0.02 | 5.47 ± 0.08 | 1.56 ± 0.02 | 0.32 ± 0.01 | 8.28 ± 0.18 | 1.63 ± 0.00 |
| 1.0 / 1.0 | 0.29 ± 0.01 | 5.74 ± 0.02 | 1.62 ± 0.01 | 0.30 ± 0.00 | 8.88 ± 0.20 | 1.68 ± 0.01 |
For the patterned membrane (MS membrane II) and $v_h = 0.5 \text{ ml/min}$, the flow and concentration profiles, and the derived concentration at the outlet for each time point are depicted in Fig. 6. It was revealed that the experimental retention time and selectivity are much lower compared to the values predicted by the simulation (Table 3). This may be due to a non-uniform cross-flow velocity as a result of differences in the membrane compaction and/or in the pore size between the ridges and the cavities of the membrane’s selective layer as it was discussed above.

5. Conclusions

To date only flat (non-patterned) UF membranes have been used in AF4 as micron-sized features are considered harmful for the separation. We have demonstrated that micron-sized grooves could in fact improve performance in AF4. This was shown by several means including moment analysis, physical experiments, and CFD simulations. Our results show that perpendicular grooves can increase retention, selectivity and resolution. This system could be useful as macromolecules and nanoparticles can be analyzed with lower cross-flow rates without the need to use higher spacer thickness or higher cross-flow to outlet flow ratio. This concept could be applied on any FFF system as it has been originally demonstrated by Giddings et al. for ThFFF [12].

The physical experiments were carried out with microstructured UF membranes fabricated by hot-embossing. This fabrication process caused an increase in the actual MWCO of the membrane (as indicated by the AF4 experiments) but the effect of the grooves could be shown with the larger protein standards used in this study (apoferitin 443 kDa and thyroglobulin 669 kDa). However, this concept could be particularly useful for smaller macromolecules, and therefore future work should be focused on the fabrication of microstructured membranes with lower MWCO and high water permeability. This could be achieved, for instance, by methods other than hot embossing such as phase separation or additive technologies (e.g., 3D printing of a non-porous or porous material over an UF membrane). Additional research with CFD experiments of different groove shapes and dimensions is underway to investigate the optimal groove structure.

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