Purification of Proteins and Nanoparticles by Continuous Field-Flow Fractionation
M. Marioli

Summary

The present dissertation investigates ways to increase sample loading and throughput in the separation technique asymmetrical flow field-flow fractionation (AF4). AF4 is a very flexible technique that separates nano-sized solutes based on their hydrodynamic size. It operates under very mild conditions because it takes place in an open structure without packing material. It has been applied extensively for biomolecules and (bio-)pharmaceuticals, however, mainly as an analytical tool since during separation the sample is compressed against an ultrafiltration membrane, hindering the injection of high mass loads. Occasionally it has been used as a preparative tool (only in lab-scale) using AF4 channels with larger dimensions. The motivation of the study is stated in **Chapter 1** and lays in the preparative purification of (bio-)pharmaceuticals whose quality is determined by the narrow size/molecular weight distribution of the final product. For example, the formulations of protein-based therapeutic agents produced by modern biotechnology (such as antibodies) need to be free of aggregates, which is typically achieved by using chromatography. Nevertheless, packed column chromatography increases substantially the production costs and there is a quest for alternative preparative purification methods.

In **Chapter 2** the historical background, the theory, and the advantages of AF4 compared to size-exclusion chromatography (SEC) are discussed. AF4 has been proven very useful for the analysis of biopharmaceuticals and complex biological samples which they may experience non-specific adsorption on the support of packed chromatography columns. It has a higher upper limit in the size range compared to SEC and therefore sample filtration is not necessary. The cross-flow can be adjusted and programmed to separate the sample components of practically any size distribution. Experimental results are shown which demonstrate the flexibility of this technique separating globular proteins with hydrodynamic size ~7-30 nm, nanoparticles with hydrodynamic size 30-100 nm, and blood plasma which constitutes of components of a very broad size range (7-1000 nm). The only limitations that have been reported in AF4 are mass overloading and, in some cases, interaction with the membrane. These factors are very important since they affect yield and throughput in the preparative use.

To investigate the factors that contribute to sample loss and overloading, a systematic study was carried out in **Chapter 3** comparing five globular proteins with different molecular weight (36.7 kDa - 669 kDa) and isoelectric point (4.0 – 6.5), and membranes of different chemistry, i.e., regenerated cellulose (RC) and polyethersulfone (PES), and molecular weight cut-off (MWCO). It was revealed that, under physiological ionic strength conditions, sample loss was dependent on the

membrane chemistry (RC had lower protein adsorption) and the membrane MWCO which was higher than its nominal value. Furthermore, the overloading effects (i.e., increase in retention time and peak broadening) were not only dependent on the protein concentration on the membrane as expected but also on the protein standard; the overloading effects were more pronounced for γ-globulin presumably due to the increase in the local viscosity close to the membrane because of the high protein concentration during sample focusing and elution. These findings indicate that the sample loading could increase significantly with changes in the composition of the carrier liquid (additives, ionic strength, pH, etc.) that aim to reduce the dependency of the viscosity on the protein concentration.

Another effective way to increase sample loading and throughput is by developing a continuous two-dimensional (2D) AF4 system. To achieve continuous fractionation, a second displacement process should occur at a right angle in which the solutes have a different selectivity compared to AF4. To this end, in **Chapter 4** microstructured (MS) membranes are introduced with grooves on their surface in order to tune the selectivity in AF4. MS membranes, fabricated by hot-embossing of commercial PES membranes, were placed in an AF4 channel with the grooves perpendicular to the channel flow and showed a significant increase in selectivity between a protein monomer and a dimer. Although the permeability of the smaller proteins used in this study was increased after hot-embossing, larger proteins such as apoferritin (443 kDa) and thyroglobulin (669 kDa) exhibited high recovery. CFD simulations supported the results and theoretical equations, based on moments analysis, were derived which relate retention time, selectivity and plate height to the groove height. Both theory and physical experiments showed that perpendicular grooves increase retention time, selectivity and resolution in AF4.

In **Chapter 5** a continuous 2D-AF4 system was developed by placing the MS membranes diagonally to the channel flow in a modified AF4 channel with multiple channel outlets. The slanted grooves are causing a lateral displacement of the solutes and the deflection angle depends on the hydrodynamic size. The system may be considered as 2D since the spatial separation occurs because of the simultaneous differential displacement along and across the grooves where the solutes have different selectivity. The spatial separation was demonstrated using model proteins (apoferritin and thyroglobulin) and PS-latex nanoparticles (with diameters of 34 and 102 nm). Next to that, the ability of the system to operate in a continuous manner for several hours was demonstrated with model proteins under an uninterrupted sample flow. A continuous system does not only have the advantage of higher throughput but also less dilution, and it is easier to automate and integrate with other continuous platforms. For this reason, it would be suitable either for preparative purification or for integration into microfluidic devices.

Finally, the possibilities of optimizing, upscaling and using of this continuous 2D-AF4 system in industrial purification, and the future in general of 2D-FFF are discussed in **Chapter 6**. Optimal microstructured membranes, having low MWCO and grooves with sharper corners, could be fabricated with alternative methods such as phase separation and additive manufacturing. A feasibility study is shown by printing an ink based on polysulfone (PS) over a RC membrane with aerosol jet printing. Lines of controllable dimensions were created but this process was more time-consuming and less cost-effective compared to hot-embossing. CFD and dimensionless analysis are great tools that can be used to optimize groove structure and channel geometry. Upscaling of the system would be achieved with a moderate increase in channel dimensions and operation in lower cross-flow rates. It is anticipated that the biggest challenges in upscaling and using this system in the production of biopharmaceuticals would be the fabrication of large MS membranes and all the procedures that need to be established (process validation, process control, microbial control, etc.).