Asymmetric Flow Field Flow Fractionation: Current Status, Possibilities, Analytical Limitations and Future Trends

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The development and applications of asymmetric flow field flow fractionation (AF4) are outlined in comparison with older and better-known size exclusion chromatography (SEC). The recent advances in AF4 instrumentation and prediction of further progress of the technique is given.

Key words Asymmetric flow field flow fractionation, size exclusion chromatography

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Asymmetric flow field flow fractionation (AF4) is undoubtedly the most developed and most widely used sub-technique from various field flow fractionation (FFF) techniques. AF4 is a variation of flow FFF and the method dates back to the paper by Wahlund and Giddings [1]. They theoretically described and experimentally proved the possibility of separating dissolved macromolecules and dispersed colloidal particles in a thin ribbon like channel having one semipermeable wall and creating a cross flow by splitting it from the channel flow. During the first step, the sample is focused in a thin band with a concentration distribution created by the balance of the diffusion coefficients and the cross flow. Smaller and faster diffusive components are on average farther away from the membrane compared to bigger ones. During the subsequent elution step, they are placed in different velocity streamlines in the longitudinal flow leading to a retention time depending on size. The main advantage of AF4 is the flexibility and versatility given by the possibility of using various cross flow profiles similarly like using various solvent gradients in high performance liquid chromatography (HPLC). Hollow fiber flow FFF (HF5), which was introduced at the same time [2], is a viable alternative [3] for specific applications [4]. Compared to AF4, the technique currently plays a marginal role. It might become more important if more routine, especially pharmaceutical, applications appear.

Since its introduction, the AF4 method has coexisted with the older and markedly more used size exclusion chromatography (SEC). Although both separation techniques have many similarities, the fundamental difference is given by the absence of a stationary phase in the AF4 channel. It is exactly the lack of stationary phase that gives the method several advantages over SEC. First of all, various interactions that often disturb the size restricted permeation in SEC [5] are eliminated or at least reduced. It is the absence of a stationary phase that confirmed the idea of anchoring of the branched macromolecules in the pores of SEC column packing [6] and which permits the determination of the true relation between the root mean square radius and molar mass and thus proper characterization of high molar mass branched polymers [7,8]. The semipermeable membrane cannot be considered to be a stationary phase despite the fact that the sample is concentrated near the membrane surface. The macromolecules do not permeate into the membrane and do not separate by steric exclusion as in the case of SEC, and the enthalpic interactions, if any, are not the primary separation mechanism as they are in the case of HPLC. However, various interactions and overloading effects are the main limitations of the applicability of AF4 especially in the case of particle separation. Strategies to minimize and overcome these limitations by proper choice of carrier solution, membrane type and cut-off, and sample preparation are discussed in the literature [9,10].

Compared to SEC, the AF4 separation is markedly gentler with respect to possible sample degradation by strong shear forces in the stationary phase. This allows the determination of the true molar mass distribution of polymers containing ultra-high molar mass fractions which undergo shearing degradation in SEC columns [11] or polymers containing nanogels that can be completely absorbed [12]. The applicability of the technique has been also demonstrated in the area of high temperature analysis of

polyolefins [11], though the high temperature resistant membranes of sufficiently low cut-offs are still unavailable.

With the advent of nanotechnology, AF4 has evolved into an important characterization technique for nanoparticles, the traditional domain of dynamic light scattering (DLS) and single particle methods like nanoparticle tracking analysis or electron microscopy. Particle separation by flow FFF was pioneered in environmental research [13], and was further adapted for metal [14] and other nanoparticles [15], and latex particles [16]. At the beginning of the millennium AF4 was first used in pharmaceutical applications for proteins and particles for drug delivery [17], then followed by the work on liposomes [18], viruses and virus like particles [19], drug carriers [20], and extracellular vesicles and exosomes [21]. With the current paradigm shift in pharmaceutical science towards gene delivery, AF4 is considered a core characterization technique to provide size distribution and drug loading efficiency of drug formulations which depend on a nanoparticle carrier [22–24].

During its existence the AF4 technique underwent significant instrumental development from laboratory-made devices to commercially available, reliable and relatively easy to operate instruments. Especially the AF4 set-ups integrating the HPLC systems of renowned manufacturers benefits from the efforts into the development of high-performance pumps, autosamplers and highly sensitive detectors. Although AF4 theory permits the determination of hydrodynamic radius from the retention time, significantly more information can be obtained by direct determination of molar mass and size by a multi-angle light scattering (MALS) detector, which can be additionally completed by the embedded DLS, and thus the recent developments of these detectors with regard to their sensitivity, precision, accuracy, simplicity of operation and of processing software is an essential part of the development of AF4.

The negative consequence of the absence of a stationary phase is that the efficiency expressed by the height of theoretical plate cannot be significantly increased to the extent witnessed over the past decades in SEC. The SEC stationary phase developed from soft particles of several tens micrometer size packed into 120-cm columns of low efficiency to nowadays high performance columns of typically 30-cm length packed with rigid 3, 5 or 10 micrometer particles and often mixing different pore sizes into a single column. With the limited efficiency, the AF4 resolution can be increased solely by increasing selectivity which generally requires analytical times to some extent longer than usually needed for a standard SEC analysis. Compared to SEC, AF4 has generally lower efficiency and higher selectivity and thus the resolution of the two separation techniques is comparable. The resolution of AF4 can be also enhanced by minimizing undesirable disturbing flow effects by improving the channel design, automatically adjustable focusing position and using smooth membranes. Especially the membrane smoothness seems to be a neglected parameter even though all macromolecules or particles, no matter of their hydrodynamic size, are during their flow through the channel in close contact with the membrane with the maximum concentration at the membrane surface. It may be worth noting that large macromolecules and particles are moving within a several micrometer distance from the membrane. As the roughness of some of the semipermeable membranes is of several micrometers, the movement of large species along the channel may be disturbed by the membrane bumpiness with the consequence of irregular fractograms and molar mass versus retention time plots. In addition, the type of bottom frit supporting the membrane can make a big difference in the peak quality. The problem appears when the frit material porosity is not spatially uniform and have regions with a few big pores. This can affect the uniformity of the cross flow field and result in wiggly peak shapes. Except for the membrane roughness, decreasing the cut-off to the proximity of 1000 g/mol can extend the applicability of the technique to mid-molar mass polymers, especially those that cannot be properly separated by SEC due to strong interactions or high degree of branching. However, the possibility of losing an oligomeric part of disperse polymers is and will remain a certain limitation of the technique. The lack of suitable membranes currently hinders the technique from analyzing polymers soluble solely in highly polar organic solvents such as dimethyl sulfoxide and thus the development in this direction can further extend the AF4 application area. For some types of samples, the resolution can be also increased by applying an electric field together with the flow field. The combination of the fields, which was recently introduced into commercially available instruments, can improve the resolution and also bring additional information about the electrical properties of samples under investigation [25].

Longer retention typically employed in AF4 usually results in the concentration of molecules eluting from the AF4 channel several times lower compared to SEC. This may affect detectability of minor components present in the analyzed samples and so the recent development of dilution control modules bypassing the sample-free part of carrier flow to waste and thus increasing sample concentration flowing through the detectors improves the detection of environmental colloids, nano-plastics, various biological samples, and ultra-high molar mass fractions and nanogels in polymers. The increased concentration together with precise flow regulation promotes the use of online viscometers that allow deep structural studies of synthetic and natural polymers and which can be also successfully applied in protein research.

In principle, the solvent consumption in AF4 is higher than in SEC as the channel flow goes together with the cross flow. This can be at least partly counteracted by returning carrier from the cross flow outlet, which contains no or trace amounts of analyzed samples, back into the carrier reservoir using intelligent solvent recycling. Such devices, which are becoming available in the new generation of the AF4 instruments, can decrease analytical costs and environmental aspects of especially organic AF4.

The channels allowing easy membrane replacement, smooth low cut-off membranes, easy to operate instruments capable of self-diagnosis and software permitting prediction and easy control of operational conditions will contribute to the popularization of the AF4 technique, which will remain the leading technique from the entire FFF family. The method will coexist with SEC with the mutual application ratio continuing to be in favor of SEC. However, the advantages of AF4 will drive the technique to many laboratories using so far SEC as the only separation technique, especially those dealing with ultra-high molar mass, branched and functional polymers. The ability of the two techniques to share the same HPLC and detector systems, and AF4 modules simply switching one separation mode into another shall contribute to this trend. In addition, one can expect AF4 to be more used in the research areas where SEC completely fails, i.e., separation and characterization of polymeric nanogels and assemblies, liposomes, nanoparticles, single-chain nanoparticles, extracellular vesicles, gene vectors, cellulose nanocrystals, various drug carriers, and environmental colloids. In the field of nanoparticles, AF4 is placed to become a main-stream technique moving out of the niche it has occupied in the last decades. This will further enhance development of instrumentation and software to make AF4 more accessible to a wide user base.

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