Chapter 15
Asymmetrical Flow Field Flow Fractionation: A Useful Tool for the Separation of Protein Pharmaceuticals and Particulate Systems

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Abstract The focus of this chapter will be on asymmetrical flow field flow fractionation (AF4) for the separation and characterization of protein pharmaceuticals and particulate systems. The chapter will provide some background and historical information on field flow fractionation and the general working principle. In addition, a practical guide on how to use AF4 will be described and critical parameters for the development of a suitable separation method will be discussed. The use of AF4 for protein pharmaceuticals as well as particulate systems will be described and some examples given in the literature will be presented. Finally, a summary of the most recent trends in AF4 and an outlook will be given for potential application fields in the future.

Keywords Asymmetrical flow field flow fractionation • Protein pharmaceuticals • Separation

1 Introduction and General Principles of AF4

Asymmetrical flow field flow fractionation (AF4) is the most important representative of a group of separation technologies developed in the 1960s following one common principle, the field flow fractionation. Separations are carried out within a rectangular, flat channel where perpendicular to a laminar flow from inlet towards outlet a second field is applied. This field can be a temperature gradient, a thermal gradient, a magnetic field, a gravity field (created by centrifugation) or a flow field (Wahlund and Giddings 1987; Schimpf et al. 2000). The perpendicular field (called "cross-flow") leads to a force that drives soluble or insoluble molecules and particles towards one of the channel walls (so-called accumulation wall). Under

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laminar flow conditions, the flow velocity across the channel thickness has a parabolic profile with the highest flow velocity at the center of the channel and slowest velocity at the walls. Smaller species diffuse back towards the middle of the laminar flow channel faster than larger species. At equilibrium, a steady state is established for each sample species at a certain distance from the channel wall. The mean thickness of this sample equilibrium layer is correlated to the retention time and physicochemical properties of the sample (Williams and Giddings 1994). By that, smaller species are eluted earlier, and when a detector is placed at the end of the separation channel, a fractogram similar to a chromatogram, can be established. Field-flow fraction (FFF) developed rather slowly in the first decade after its introduction in 1960 (Wahlund and Giddings 1987), and it lasted until about the year 2000 until the protein formulation community took real notice of the method (Klein and Huerzeler 1999; Fraunhofer and Winter 2004). This has several reasons, one of them being the fact that - at that time- a small number of suppliers had optimized AF4 systems so far, that they were commercially available for a reasonable price and could be used after a few hours of training by experienced HPLC users. This went along with the development of the asymmetrical version of flow field fractionation, that makes the systems much easier to build and to apply (Giddings 1993). Let us consider how a flow field is built up in a channel with laminar flow: The upper and lower wall of the channel have to be replaced by semipermeable membranes that allow liquid to be pumped into the channel and (on the other side) to be removed to create the cross flow (Fig. 15.1).

Such systems had been built e.g. by Giddings, and even though they worked very well (see application examples for liposomes, lipoplexes under 3.2), their setup was rather cumbersome. In a next step, the upper semipermeable wall was removed and

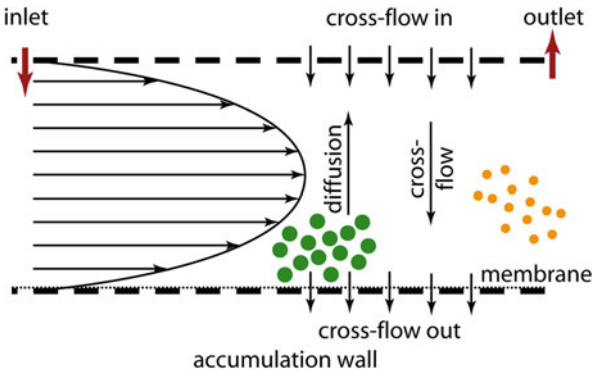

Fig. 15.1 Schematic illustration of the separation/fractionation principle of AF4. The parabolic flow profile of the channel flow transports the sample injected through the inlet through the AF4 channel. The cross-flow perpendicular to the channel flow forces the sample towards the accumulation wall. Separation/fractionation is then a result of two parameters taking place simultaneously. Smaller particles will diffuse back faster towards the channel center due to Brownian motion compared to larger particles. Secondly, particles located close to the accumulation wall will start to rotate and, as a consequence, will experience hydrodynamic lift forces back to the channel center

the cross-flow only achieved by pumping out water on the lower channel wall. Although the field would then be imperfect and "asymmetrical", it serves the purpose perfectly as the movement of the analytes and their separation takes place close to the outlet wall and therefore the imperfection of the upper part of the channel plays no major role. This makes the systems finally cheaper and easier to handle which catalyzed its success (Wahlund and Giddings 1987).

Another driver for the increased interest in AF4 was the fact that in protein formulation sciences the relevance of aggregates and small, subvisible particles became more and more obvious, and particulate and nanoparticulate drug delivery systems needed more reliable analytics. As we will see in detail, AF4 can close certain gaps that other methods leave open, especially when size exclusion chromatography (SEC) as the standard for soluble macromolecules and optical methods measuring nanoparticles are considered. Before we discuss the method in more detail, a recent technical development shall be introduced. Hollow fiber FFF (sometimes called HF5) is the logical consequence when the AF4 principle is brought to its theoretical optimum. Why not transforming a channel with one semipermeable wall into a round tube where the entire wall surface can be used for removing liquid and creating a flow field? Existing technologies from hollow fiber dialysis modules were adapted to AF4 and the new "HF5" system was created (Reschiglian et al. 2014; Zattoni et al. 2008). With that, FFF reached a grade of convenience similar to column based chromatographic systems, where the user "plugs in" the separation device (now the "HF5 hollow fiber module") and starts the run.

2 Practical Guide for AF4

Before carrying out a separation of a sample with AF4, we shall consider first the reasons to do so, namely the potential benefits over other methods and then how to run a sample and how to interpret the resulting fractogram. Of course, over the past decades excellent reviews have been written and handbooks edited, amongst which the books by Schimpf, Caldwell and Giddings are still considered as standard monographs (Schimpf et al. 2000; Caldwell 1988). The core of the AF4 method is the separation channel; all other peripheral compounds are comparable to standard chromatographic systems, be it the autosampler or the numerous detectors that can be used in line, like UV/VIS spectrophotometer, fluorimeter, refractive index (RI) detector, or multi-angle laser light scattering (MALLS) detector. Compared to a chromatographic column, an AF4 channel is first of all empty. No large surfaces, as they are typical for the column fill material, are provided and no inlet/outlet frits are needed. At least in theory, anything can be brought into the channel that is smaller than the channel height and everything leaves the channel eventually. Typical (inner) channel dimensions are a length of 10–40 cm, a width of 1–5 cm and a height of 100–500 μm (with more typically a height of 250–350 μm). With that, almost all types of protein aggregates and pharmaceutically relevant nanoparticles, even small microparticles can be loaded on an AF4 channel. Upper

size limits as for the standard SEC do not apply anymore. It is well known that due to sample filtration and the frit inlet of the SEC columns, one will always see only the soluble part of potential impurities and large species are systematically lost or underestimated. Thus, AF4 has a clear advantage over SEC in this respect. AF4 can also be used in a semi-preparative mode by applying larger channels. On the other hand, AF4 has a lower limit for molecules that can be analyzed. The cut-off of the semipermeable membrane that closes the channel is the limiting step. Cut-offs of about 10 kDa are standard. For smaller analytes one could use smaller cut-offs, like e.g., 5 kDa, but pressure in the system then rises and finally losses for molecules in size close to the cut-off make quantitative analytics impossible. Insulin may be named as the molecule marking the lower limit of AF4. As the channel is empty and unspecific to the type of analytes, they are only separated by their hydrodynamic radius, not by any other physical or chemical feature. However, certain molecules may bind unspecifically to dialysis membranes and therefore the general separation principle of AF4 may be somewhat compromised in these cases. Another feature of AF4 separation is that in practically all cases, the formulation in which the drug substance is dissolved can be used as a running buffer. This ensures that especially for aggregation phenomena, changes in pH, ionic strength, buffer type, surfactant concentration, and concentration of stabilizers that all could influence the analytical result can be avoided. As mentioned above, adsorption to the cross-flow wall can occur, and in such cases addition of surfactants or salts may be necessary. But molecules of this type often adsorb to other surfaces, too, and must be formulated with surfactant anyway to avoid losses during pharmaceutical fill and finish operations. Reschiglian et al. provided a concept that eliminates the membrane, but this could only be applied to very large particle/colloids in the sample and has therefore not found broader application (Reschiglian et al. 2000). Another argument that has been used in favor of AF4 versus SEC is the fact that the shear forces applied to a molecule or a colloidal particle are much lower in AF4 than in SEC. Higher overall pressure and the narrow spaces between the densely packed gel particles in an analytical column speak in favour of AF4 and data received from analytical ultracentrifugation (AUC), SEC and AF4 confirm this view in a way that aggregates are underestimated by SEC because they are “milled” down to smaller oligomers or dimers. On the other hand, AF4 is often criticized for its potential to artificially create aggregates. The main reason for that is the “focusing step” that is needed before an AF4 separation is started.

2.1 The Focusing Step

A sample injected into the running buffer of an AF4 channel would spread in a way that only weak, flat peaks will be achieved. To avoid this, after injecting a sample into the inlet area of the channel, forward flow and backflow are applied and the entire running buffer leaves the channel through the cross-flow membrane (Fig. 15.2). In that phase, a sharp band of the sample species is formed, preferably

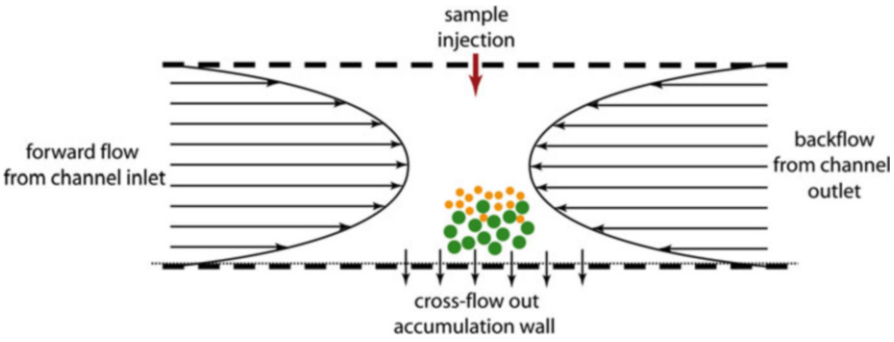

Fig. 15.2 Schematic illustration of the sample positioning during the focusing step applied in AF4. The flow from the channel inlet and the inversed flow from the channel outlet force the sample within a narrow band. Particles arrange at a distance to the accumulation wall according to their diffusion coefficient

close to the inlet side of the channel. Finally, the backflow is stopped and the regular separation starts with a forward flow plus a cross-flow. During the focusing step, the sample is concentrated in a band close to the membrane and under these conditions one could imagine that the higher concentration in the focus band and the microfluidics moving the molecules towards the membrane surface may induce aggregation. Control runs, if possible, are needed to exclude such artifacts (Litzén and Wahlund 1991).

2.2 Instrumentation

Before we consider performing (as a thought experiment) an AF4 run, we need to choose an instrument. The market is rather clearly laid out, as there are only two major competitors in Europe and North America. Both provide the user with excellent systems for more than 10 years; however, their technical philosophy is slightly different. Wyatt Technology (Wyatt Technology Europe GmbH, Dernbach, Germany), uses standard HPLC compounds for dosing the running buffer, for injecting the samples, and creating the linear and cross-flow; in fact, only a single pump is used. Whilst theoretically any system could be used, in practice the compounds are taken from Agilent. To exactly control the different flows, a "switchbox" with valves, pressure sensors, splitters etc. is used, called "Eclipse" system by Wyatt. Postnova (Postnova Analytics, Landsberg am Lech, Germany) uses a different approach. Several pumps provide the different flows and they are all custom-built as are the autosamplers, controllers etc. Both companies provide several detectors, with Wyatt having an emphasis on MALLS detectors. Due to the fact that Wyatt has built and optimized MALLS detectors and pertaining evaluation software systems for some decades, they rely on the SLS software package ASTRA but control the AF4/HF5 systems with a different software.

Postnova has one software package comprising fractionation run control and SLS evaluation, but has less SLS experience overall (Wyatt 1991; Roessner and Kulicke 1994).

MALLS detectors are common and relevant for AF4 applications, as they allow a direct and independent determination of the molecular size of an analyte. This is helpful and often used in SEC, too, but in AF4 two more aspects have to be considered. Firstly, retention times in AF4 are less easy to calibrate and to predict compared to the situation in SEC, therefore an independent method is needed. Secondly, the chance to have very large molecules or colloids running through the AF4 channel that cannot be separated by other methods so easily makes MALLS detection very attractive to estimate their size/molecular weight immediately in line (Wyatt 1991; Roessner and Kulicke 1994).

2.3 *Separation*

If we start an AF4 run, we first need to place the membrane into the separation channel (Fig. 15.3), equilibrate it with the running buffer, rinse the system and inject the sample. After focusing the sample band, the separation can start. For a given channel and membrane (only regenerated cellulose and polyethersulfone are on the market) the linear flow and the cross-flow are the only variables. The volume of the cross-flow enters the channel together at the inlet with the volume that finally leaves the channel at the outlet. Considering this, one becomes aware that the sample is diluted over the course of the separation run. Part of that is counteracted by the "up-concentration" during the focusing step. Another trick allows increasing the concentration of the analyte entering a detector after the channel. This feature is called "slot outlet" and uses the fact that the separation takes place slightly above the accumulation wall and the rest of the channel height is almost free of sample molecules. Consequently, only the part of the channel content next to the

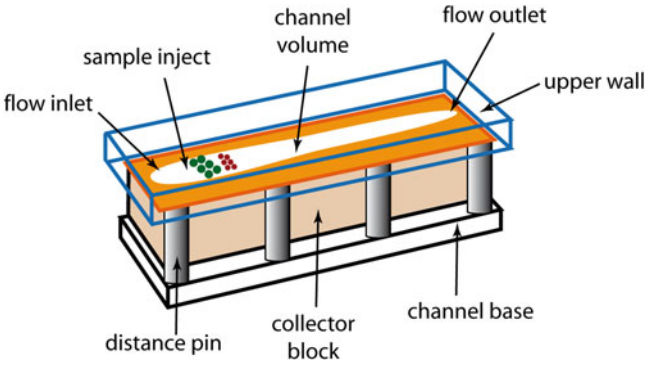

Fig. 15.3 Schematic drawing of a typical AF channel. Following sample injection and focusing samples are separated. Smaller particles (*red*) elute prior to larger particles (*green*)

accumulation wall is taken to the detector ("splitting" the flow), the rest is discarded. Before the optimal cross-flow is selected, a run without cross-flow is helpful to determine whether the sample is retained by the separation system as such, e.g., by adsorption to the membrane, and furthermore what molecule sizes are present in the sample before it has seen focusing and cross-flow. In case of strong membrane binding, albumin can be used to block such adsorption. Now the cross-flow is selected. Typically a cross-flow is not kept constant over the entire separation run but reduced over time (like a gradient) and finally set to 0 at the end. It is therefore a typical feature of an AF4 run, that all sample material that has been accumulated and not rolled towards the outlet according to the equilibrium distance from the wall, will then be released and detected as a wash off peak.

Larger species tend to elute not earlier than with this peak, and care has to be taken not to overestimate such species because of aggregates having formed at the accumulation surface and not being present in the sample as such. Theoretical calculations are possible to estimate the retention time of molecules or nanoparticles under given conditions (channel dimensions, fluxes, densities, viscosities, temperature) and allow indirectly to identify interactions that disturb the theoretical ideal diffusion behavior due to surface interaction and/or self-interaction of the analyte species (Giddings 1978).

In summary, the development of a suitable AF4 experimental set-up requires time and analysis of the various parameters having an influence on the quality of the resulting AF4 fractogram. Critical experimental parameters are the ionic strengths of the carrier liquid, the focus flow rate, the cross flow rate and the sample load. Some parameters may be more critical than others, but nonetheless, development of an appropriate AF4 method is time-consuming and non-trivial (Williams et al. 2001; Williams and Giddings 1994).

2.4 Steric Mode

Very large species within a sample show the so called "steric mode" elution behavior and elute in front of all the rest with the injection peak (Fig. 15.4). Although such steric mode pre-peaks are not regular shaped and not resulting from a quasi-equilibrium situation, they can deliver important qualitative information (Caldwell et al. 1979). The presence of very large species like oil droplets, agglomerates and particles can be confirmed in one run, a feature that SEC will not be able to deliver. Fraunhofer and Winter have shown that the presence of silicon oil droplets from prefilled syringes can be differentiated from protein drug aggregates (Fraunhofer and Winter 2004; Fraunhofer 2003). Gottschalk et al. went one step further and used the steric mode pre peak to quantify very large, particulate protein aggregates whereas the soluble, large aggregates run under normal separation mode (Gottschalk et al. 2006).

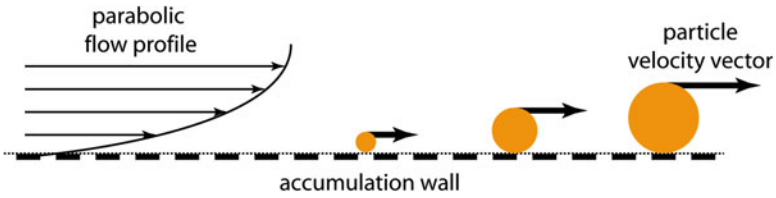

Fig. 15.4 Schematic illustration of the steric mode principle in AF4. Larger particles possess a higher particle velocity vector and are therefore eluted earlier compared to smaller particles from the AF4 channel

2.5 Benchmarking of AF4

2.5.1 Comparison to Electron Microscopy

Electron microscopy is widely used for the characterization of systems in the nano- and micrometer range. Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and both cryo-SEM and cryo-TEM are currently utilized. Despite their advantages to fully elucidate the surface structure of a system, disadvantages are the tedious sample preparation and the time-consuming sample analysis. In addition, only a small fraction of the total sample can be analyzed. While in cryo-SEM and cryo-TEM samples are viewed in their frozen state, SEM and TEM usually require a drying and/or staining step, which may alter sample morphology and produce artifacts. In contrast to this, AF4 allows the analysis of a larger fraction of the total sample, gives detailed information about particle size, allows fractionation of different particle size fractions, and furthermore nanoparticles can be analyzed in liquid dispersion.

2.5.2 Comparison to Size Exclusion Chromatography

Size exclusion chromatography (SEC) is the standard method for the analysis and separation of proteins and other polymers of pharmaceutical relevance. Being the workhorse in every biopharmaceutical lab, SEC has matured to a very reliable and precise method. Qualification of the equipment and validation of the methods are common practice (Arakawa et al. 2010; Philo 2009). Still, SEC has its limitations and drawbacks. First, columns are very expensive, their standing times are often frustratingly short, especially when samples are rich in impurities, and the columns have to be selected for the samples under investigation, meaning that one needs different columns to cover a broad range of analytes. AF4 has certain advantages here. As already mentioned, one channel serves for the separation of all types of analytes from 5000 Da to particles of several hundreds of nanometers. Furthermore, is has been shown that SEC underestimates larger protein aggregates compared to AF4. For that reason, since the 1990s, AF4 received increasing interest as a new tool (“orthogonal method”) for protein, nucleotide and polymer research (Litzén

and Wahlund 1989; Litzén et al. 1993; Gabrielson et al. 2007; Carpenter et al. 2010). When it comes to the quantification of fragments, AF4 might have its weaknesses as small compounds can get lost due to the chosen membrane cut-off, but typical fragments formed from antibodies will be well retained, separated and detected. AF4 has therefore long been used as a trouble-shooting device and research instrument only, but now the first systems run under current good manufacturing practice (cGMP) conditions, and AF4 methods are used for the first products and projects for regulatory specifications.

2.5.3 Comparison to Analytical Ultracentrifugation

Although AF4 has shown its promises over SEC for larger particles and protein aggregates, AUC is often chosen when a method is sought where the interaction with the analytical separation systems can be reduced to practically zero and artifacts are very unlikely (Pauck and Coelfen 1998). Due to the mandatory presence of the channel membrane, AF4 can as a matter of principle not provide that. AUC is extremely expensive, needs experienced operators and specialists for interpretation, and by that, is not useful as a routine method accompanying product development, clinical supplies, scale up, and product release. In our eyes AUC should be used to control SEC and AF4 for their ability to detect relevant amounts of aggregates or colloids. Once qualified in that way, AF4, or in case of "easy" samples also SEC, could then be used for routine purposes.

3 Analysis of Particulate Systems

3.1 Nanoparticles

As described above, AF4 is a versatile tool for the analysis of various types of formulations. However, in particular for analytics of nanoparticulate formulations or particulates, it has been shown in various reports in the literature that AF4 offers additional attributes when it comes to sample analysis. Usually, nanoparticulate formulations are characterized by the classical analytical methods such as electron microscopy and dynamic light scattering (DLS). Electron microscopy allows investigating the surface of the nanoparticles, whereas analysis in terms of particle size and size distribution is cumbersome, since at least 10,000 particles must be counted in order to satisfy statistical requirements. DLS on the other hand does not provide any information on the surface characteristics of the sample, but can be utilized to calculate an average particle size and a size distribution according to the Stokes-Einstein equation. However, this technique is in principle only applicable to monodisperse formulations that contain perfectly spherical particles at an ideal concentration. AF4, on the other hand, allows obtaining information on particle size

while at the same time particles can be fractionated according to their size, should particles of different sizes be present in the formulation. In the following section an overview over different nanoparticulate formulations investigated by AF4 is given. A nice tutorial on how to select and develop a protocol for nanoparticle fractionation by AF4 can be found in Gigault et al. (2014a).

3.1.1 Human Serum Albumin Nanoparticles

John and Langer (2014) analyzed 150 nm human serum albumin (HSA) based nanoparticles in terms of particle size but also investigated the particle formation process using AF4. The authors report that the determination of an ideal cross flow for sample analysis is the main influencing parameter on the separation quality. Therefore, in a first step the cross flow should be varied. Cross-flow should be sufficiently high for particle separation, but at the same time as low as possible to achieve an optimal peak shape in the AF4 fractogram. In addition, the focusing step is highlighted as an important parameter. If the focusing step is too short or the focus flow too low, particles will not be fractionated completely. On the other hand, if the focus flow and the cross flow are increased to such an extent that the particles are located too close to the membrane, more time is needed for complete elution. In terms of particle concentration for AF4, a compromise needs to be found between a starting concentration that is sufficiently high (as the sample will be diluted during fractionation) but not too high since otherwise overloading effects of the channel will occur.

In addition to the analytical method for particle size characterization described above, the authors also utilized AF4 to observe HSA nanoparticle formation during the desolvation process, as well as characterized the size and degree of PEGylated HSA nanoparticles. Increasing amounts of ethanol were added for HSA desolvation which first resulted in the formation of protein aggregates and later in the formation of HSA nanoparticles. Covalent modification of HSA nanoparticles with PEG was observed by an increase in particle size and a time shift of the particle count rate. According to the author's reports, AF4 can be used to also quantify the degree of PEGylation when unbound PEG is previously removed from PEGylated nanoparticles.

3.1.2 Gelatin Nanoparticles

Fraunhofer et al. (2004) were able to show that AF4 is a powerful tool to characterize gelatin nanoparticles in addition to the characterization of gelatin bulk material and analysis of the nanoparticle drug-loading process. Using AF4, the authors were able to separate and quantify e.g., proteins and oligonucleotides, which were even more challenging, since the raw material of the nanoparticles was also of a proteinaceous nature. In addition, AF4 allowed confirming the heterogeneity of the gelatin molecular weight. In a further study, Zillies

et al. (2007) utilized AF4 to quantify the PEGylation efficiency of nanoparticles without further sample preparation.

3.1.3 Particles from Melt Extrusion

AF4 has also been reported as an analytical method to investigate and characterize the formation of structures or particles when solid dispersions prepared by melt-extrusion were dispersed in aqueous media (Kanzer et al. 2010). Kanzer et al. compared the size and size distribution obtained by either PCS or AF4 coupled with an online MALLS detector. In contrast to PCS, which only indicated submicron particles, AF4 allowed separating up to three different nanoparticulate fractions: colloidal polymer, nanoparticle aggregates and nanoparticulate aggregates of the incorporated drug (Kanzer et al. 2010), therefore providing a much deeper insight into the bulk phase of the dispersion compared to PCS.

3.2 Liposomes, Liposomal Formulations and Lipoplexes

Apart from solid nanoparticles, liposomes, liposomal formulations and lipoplexes have also been investigated. In the early days, classical flow field flow fractionation (FFF) has been employed to investigate the size and size distribution of liposomes. In 2006, Hupfeld et al. (2006) reported on the analysis of small liposomes. The authors used three different techniques to analyse liposomal size, namely PCS, SEC with subsequent PCS analysis, and FFF coupled with on-line static light scattering and RI detectors. While all three methods delivered useful results, the size distribution calculated was not identical. Bulk analysis of the liposomal preparation by PCS revealed a broad mono-modal or bimodal size distribution, whereas after fractionation by either SEC or AF4 smaller liposomes with a much narrower size distribution were obtained in addition to a broader peak representing larger particles. The authors concluded that bulk analysis of liposomal formulations by PCS often suffers from the limitation that PCS tends to underestimate smaller particles when at the same time larger particles are present. On the other hand, results obtained by FFF showed larger minimum liposome diameters, which the authors explained by the different detection limits of the PCS measurements and MALLS detection. Hence, fractionation of the sample prior to size analysis can result in more reproducible particle sizes (Hupfeld et al. 2006). In a consecutive study, Hupfeld et al. (2009) utilized AF4 to systematically investigate the effect of focus flow rate, cross flow rate, sample load and ionic strength of the carrier liquid on the retention behavior of liposomes separated by AF4. Varying the focus flow resulted in peak shifts and changes in peak shape, therefore for each formulation at first an optimal focus flow should be determined. Secondly, sample load had an effect on calculated geometric radii. Overloading effects of the channel may be observed when the sample load is too high, meaning that some larger particles do not reach

their expected position within the channel and remain in proximity to the parabolic flow profile. As a consequence, such particles will then elute earlier than expected. Another important aspect is the influence of the cross flow rate and the cross flow mode. Different constant cross flow rates were applied. While too small cross flow rates result in insufficient fractionation, high cross flow rates hinder complete elution of the sample. Therefore, in most cases a cross flow gradient is applied to obtain both satisfactory separation and complete elution. Lastly, the ionic strength of the carrier solution also impacted on the fractionation and size analysis of liposomes, since the retention of charged liposomes in a low or high ionic strength carrier medium may be different to highly purified or distilled water and also compared to the fractionation of uncharged liposomes. Not only does the zetapotential of the liposomes influence the location of the particles from the channel wall; the orientation of phosphatidyl choline groups within the liposome may also change upon an increase of the ionic strength, hence impacting on the zetapotential of the particles and consequently on the repulsive effects between individual liposomes as well as between liposomes and the accumulation wall (Hupfeld et al. 2010). Again, an optimal salt concentration needs to be found for sample analysis. If the salt concentration of the liposomal formulation is different to the carrier medium, shrinking and swelling effects of liposomes due to osmotic pressure can be observed (Hupfeld et al. 2010). Apart from liposome fractionation and size analysis, AF4 has recently been used to study drug transfer from liposomal formulations, using donor and acceptor liposomes and quantifying the amount of drug retained in the donor liposomes (Hinna et al. 2014).

FFF has further been used to analyze physical characteristics such as particle size and storage stability of self-assembled cationic lipid-DNA complexes (lipoplexes). These systems usually suffer from their heterogeneity, as free liposomes, DNA, and complexed DNA-liposomes are present in one single formulation. In 2001, Lee et al. studied the feasibility of an FFF system coupled with UV, RI and MALLS detectors. Exploring different ionic strengths of carrier liquids and types of membranes, the authors were able to establish a system which enabled them not only to study such complex systems of various lipid-DNA ratios, but also revealed that with MALLS detection the formation of aggregates upon storage became visible which was not observed with other techniques such as PCS (Lee et al. 2001). In addition, cationic lipid-DNA particle fractions can be fractionated using FFF which can be useful if structure-activity relationships of the different complexes need to be determined.

3.3 Virus-Like Particles

Virus-like particles (VLPs) as vaccine carriers have also been investigated using asymmetrical flow field-flow fractionation. Lang et al. (2009) report on the analysis of nicotine-conjugated VLP carriers, where AF4 was used as a complementary technique to DLS. The aim of this study was to investigate aggregated species of

VLPs, which is not possible by DLS or by SEC. The authors were able to separate VLP formulations into VLP fragments, monomers, dimers and oligomers/aggregates. AF4 was further used in the formulation development and testing of formulation stability over time. Chuan et al. (2008) reported an optimized AF4 method for the characterization of VLPs, and the authors focused on potential adsorption effects as well as on the separation of VLP fragments, monomer, dimer, and aggregates. AF4 was benchmarked against classical techniques such as TEM and DLS. Under optimized conditions, no sample aggregation effects were observed. VLP samples could be fractionated by AF4, and approximately 69 % of the VLPs showed a size between 15 and 35 nm, whereas the remaining fraction was composed of particles having a size range up to 100 nm. In addition to this, the authors reported that using AF4 even changes in the quaternary structure of the VLPs could be monitored. In a further study, Pease et al. (2009) analyzed four different types of VLPs: VLPs with and without packaged genomic DNA or protein, VLP with packaged foreign protein, VLP with packaged genomic DNA, and VLP assembled from VLP pentamers modified to express a foreign peptide sequence on the VLP surface.

3.4 Polyplexes

Noga et al. (2013) reported on the use of AF4 to investigate hydroxyethyl starch (HES)-coated polyplexes. In this study, the authors synthesized different HES-polyethylenimine (PEI) conjugates and used AF4 to investigate the rate of biodegradation upon incubation with alpha amylase (AA). The authors were able to show that degradation of HES in the presence of AA is rapid at the beginning and subsequently levels off after 4–6 h. Furthermore, lower molar substitution of HES showed higher degradation rates. The effect of molar mass on biodegradation was also investigated, showing that larger molecular weight HES (e.g., HES70) was degraded to a higher extent compared to lower molecular weight HES (e.g., HES 30). The authors explained this result by the better accessibility of α-1,4-glycosidic bonds of HES in the higher molecular weight HES molecules for AA.

3.5 Non-spherical Nanoparticles

In contrast to classical DLS measurements, field flow fractionation and in particular AF4 can also be used to analyze, quantify and separate particulate systems which are not perfect spheres, for example protein filaments or non-spherical particles. In 2004, Jores et al. (2004) reported on a study using symmetric flow field-flow fractionation to compare size and size distribution of solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) with DLS measurements. The colloidal structures were prepared from glyceryl behenate and medium chain

triglycerides in order to combine mixtures of solid and liquid lipids to increase drug loading and improve controlled release thereof. PCS indicated that SLN and NLC differed from a nanoemulsion with respect to Brownian motion due to asymmetric particle shapes, thereby also leading to higher polydispersity indices. The authors reported that using symmetric field-flow fractionation allows separation of isometric from asymmetric particles, as isometric particles will elute earlier. Here clearly the principle of field flow fractionation has great advantages compared to DLS in bulk, since isometric and asymmetric particles are separated before the measurement in field flow fractionation.

Further studies using classical FFF for non-spherical particles have been reported by Chun et al. (2008) for single wall carbon nanotubes (SWNTs). Here the authors characterized the rodlike structures according to their size by comparing the elution times of fractions of SWNTs to standard spherical particles.

Mathaes et al. (2013) utilized an AF4 system equipped with a MALLS detector for measuring the radius of gyration (geometric radius) and a DLS detector to determine the hydrodynamic radius of 40 nm spherical and stretched polystyrene particles. From the measurements, a shape factor (the quotient of the geometric radius to the hydrodynamic radius) can be calculated and enables to clearly distinguish spherical from elongated or rod-like particles. However, AF4 is limited in terms of particle size by the resolution of the DLS detector. In the presented study, the DLS only allowed to measure particles in the size range between 3 and 200 nm. Gigault et al. (2014b) also looked into the characterization of self-assembled nanofibers by AF4 to investigate the length of the nanofibers as well as their aggregation potential.

Care must be taken when non-spherical particles are analyzed by either FFF or AF4. Phelan Jr and Bauer (2009) highlight that up to a size of about 500 nm rodlike particles elute using FFF by a normal mechanism, whereas an inverse steric effect occurs when larger particles are separated. Alfi and Park further investigated this topic using FFF and concluded in their study that the separation behavior of rods and spheres also strongly depends on the conditions used for the fractionation procedure (Alfi and Park 2014).

4 Preparative Use of AF4

AF4 cannot only be used to fractionate and then analyze portions of sensitive samples such as proteins or antibodies, this technique can also be used to preparatively separate protein aggregates from monomer species (Freitag et al. 2011b). Separate fractions of aggregate species can then be analyzed in much more detail. Freitag et al. (2011b) utilized AF4 to separate and quantify fragments, monomer and soluble oligomers of a therapeutic antibody. The molecular weight of the detected species was calculated according to the fractogram obtained using the described AF4 method. It was possible to store the different fractions either at 2–8 °C for up to 3 weeks without any changes in the soluble oligomer fraction.

Alternatively, fractions were frozen in the deep freezer (−80 °C) and subsequently gently defrosted in the refrigerator at 2–8 °C without any changes in the peak position, retention time or loss of soluble oligomer due to adsorption phenomena. In addition to the excellent protein separation potential, AF4 can also be run using different ionic strength buffers; hence samples can be analyzed using any tonicity of the formulation which may later be used for in vivo studies. In a further study, Freitag et al. (2011a) were able to show that by using a rinsing/disinfection routine for the AF4 instrument, it was possible to obtain protein fractions with endotoxin levels far below the required 0.250 IU/mL for parenteral administration according to the monograph "water for injections" in the European Pharmacopoeia. This semi-preparative approach has not been published before, except for a circular AF4 variant (Maskos and Schupp 2003).

5 Recent Trends in AF4

In the following section, the most recent trends in using AF4 are summarized. The examples given here highlight that AF4 receives increasing attention from researchers not only in the pharmaceutical field, but also in various other disciplines and various different types of applications.

5.1 Quantitative Characterization of IgG Aggregates

Protein aggregates in pharmaceutical formulations receive growing attention due to the potential risk of aggregates to induce unwanted immunogenicity in patients. While a number of techniques is available for protein formulation characterization (SEC, DLS), it is recommended to utilize orthogonal methods for protein aggregate characterization such as AUC or AF4 (Carpenter et al. 2010). Ma et al. recently studied soluble aggregates formed in heat-stressed solutions of an IgG molecule to which polyacrylates were added for stabilization purposes (Ma et al. 2014). In contrast to other techniques it was possible with AF4 to obtain information on % IgG recovery in addition to quantify and size the fraction of IgG monomer or dimer in comparison to IgG aggregates.

5.2 Separation of Different Types of Antibody Aggregates for Immunogenicity Testing In Vivo

As described above, protein aggregates are linked to unwanted immunogenicity in patients. However, it is still a matter of debate if all aggregates in general are

responsible for immunogenicity, or if immunogenicity is only linked to certain types of aggregate species (soluble, insoluble, reversible, irreversible aggregates). Freitag et al. separated samples of a murine monoclonal antibody based on aggregate size by AF4, and the collected fractions were subsequently used to test immunogenicity in vivo (Freitag et al. 2014).

5.3 *Separation and Quantification of Protein Aggregates by HF5*

Fukuda et al. qualified the still not so extensively used HF5 method for the quantification of a monoclonal antibody and aggregates thereof in terms of precision, accuracy, linearity, and quantitation limit (Fukuda et al. 2014).

5.4 *AF4 as an Additional Method to Gain Insight into Degradation Pathways of Antibody-Based Drug Candidates*

Fincke et al. utilized AF4 in addition to spectroscopic techniques, DLS, differential scanning calorimetry (DSC), electrophoresis, visual inspection, and surface plasmon resonance (SPR) to investigate degradation pathways of three different antibody-based drug candidates upon exposure to elevated temperature (Fincke et al. 2014). This study highlighted the usefulness of AF4 as an orthogonal technique to classical bulk measurement techniques such as DLS.

5.5 *Characterization of β-Cyclodextrin-Dextran Polymers for Poorly Water Soluble Drugs*

di Cagno et al. (2014) evaluated the potential of different newly synthesized β-cyclodextrin-dextran polymers for parenteral administration of poorly water soluble drugs. The authors investigated the stability of the new substances in terms of aggregate formation by molecular weight, the ability to solubilize a poorly soluble drug as well as drug release properties.

5.6 PLGA Nanoparticles Released from a Tablet

Engel et al. (2014) reported on the use of AF4 as an analytical method to determine the release of PLGA nanoparticles embedded in a tablet. Two different sizes of PLGA nanoparticles (120 and 220 nm) were prepared and subsequently dried using either spray-drying or freeze-drying. This dry nanoparticle powder was then transformed into tablets by direct compaction using α-lactose-monohydrate, PEG 4000, polyvinylpyrrolidone, crosscarmellose sodium and silicium dioxide as further ingredients. Release of nanoparticles from the solid dosage forms was then analysed using AF4 without further treatment such as filtration. The authors report that nanoparticle release from the tablets was completed within 30 min, and the presence of nanoparticles in the release media could be clearly proven by AF4 as well as by DLS batch measurements. This is a very interesting approach as AF4 offers the possibility to quantify nanoparticle release and potentially released API in its soluble form by UV detection at the same time, given the concentration of the API is sufficiently high.

5.7 Characterization of Cationic Polymers for Gene Delivery

AF4 has also been investigated as a characterization technique for cationic polymers. Wagner et al. studied different cationic polymers for their applicability in gene delivery and used AF4 as an additional technique to AUC and NMR spectroscopy (Wagner et al. 2014). The authors describe that SEC and mass spectrometry (MS) often do not deliver meaningful results, in particular when cationic polymers are analyzed. For SEC, this is due to the fact that the cationic polymer strongly interacts with the stationary phase of the SEC column; therefore the authors investigated AF4 as an alternative technique since in AF4 no stationary phase is present. Nonetheless, care must be taken when choosing the channel membrane, as strong interactions with the membrane material may lead to peak deformation and broadening. Additionally, the ionic strengths of the eluent must be evaluated carefully when charged polymers are being fractionated as must be the amount of injected sample as overloading effects may occur due to electrostatic repulsion. In summary, AF4 was established as a reliable method to obtain conformational information and determine the molar mass and PDI of different polymers given that the molar mass of the polymer is larger than 15 kg mol^{-1} (Wagner et al. 2014).

5.8 *Characterization of Polymersomes Using AF4*

Till et al. investigated several polymersomes based on polyethylene block copolymers using AF4 and compared the results to classical characterization techniques such as electron and atomic force microscopy, as well as static light scattering (SLS) and DLS, and small-angle neutron scattering (Till et al. 2014).

5.9 *Quantification and Characterization of Nanoparticulate Additives in food*

In a recent study, Heroult et al. described the development of an AF4 method for the characterization and quantification of silica nanoparticles in a commercially available food product, coffee creamer (Heroult et al. 2014). The presence of nanoparticulate additives such as silver nanoparticles as antibacterial additives, titanium dioxide as whitener or silica as an anti-caking agent is widely performed in food industry. These excipients are also used in pharmaceutics, therefore, the developed AF4 method may also be relevant for further studies.

6 Conclusion

In summary, AF4 has become a technique which is more and more relevant in the pharmaceutical setting and can be used as an orthogonal method to well-established classical characterization techniques. However, method development for AF4 remains to be non-trivial and may be a time-consuming procedure. Nonetheless, in some cases, AF4 may close an open gap that other techniques cannot capture. It will be interesting to see how AF4 will further evolve and in particular, if and how further developments of e.g., the HF5 system will proceed in the future.

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