Review

Field-flow fractionation for molecular-interaction studies of labile and complex systems: A critical review

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HIGHLIGHTS

- Applications of field-flow fractionation (FFF) to study molecular interactions between various types of analytes are reviewed.
- Fragile structures and weakly-associated complexes can be preserved during FFF separation.
- FFF can be coupled to various detectors to provide structural, morphological and chemical information.
- Asymmetrical flow field-flow fractionation (AF4) can be used to study the formation of protein corona.
- For certain classes of analytes, interactions with the currently available membranes are unavoidable.

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ABSTRACT

Asymmetrical flow field-flow fractionation (AF4) has attracted considerable attention as a size-based separation technique, due to its mild separation conditions, broad working range (from approximately \(10^3\) to \(10^6\) Da molecular mass or from 1 nm to 1 \(\mu\)m particle diameter), and versatility. AF4 is primarily being used to measure particle size, polydispersity, and physical stability of various systems, such as (bio)-macromolecules and nanoparticles. In comparison with size-exclusion chromatography (packed column), AF4 (open channel) allows separation while preserving labile structures. Monitoring of interactions between different compounds and in very complex matrices is possible. Preservation of the structure and correlation of structural characteristics with activity and functionality can bolster the development of new therapeutic strategies for diseases and new materials with improved properties. In this review, a detailed overview is presented of developments in AF4 for interaction studies between various systems, such as protein-protein, polymer-polymer, nanoparticle-drug, and nanoparticle-protein. The prospects and obstacles for AF4, and other less-commonly used types of FFF, for studying...
interactions within complex and fragile systems are covered. Coupling AF4 to a variety of detection systems can greatly contribute to the understanding of the interaction/association processes and provide information on the interaction kinetics. This review is intended to provide comprehensive documentation on the types of interaction (structural, morphological, chemical) on molecular interactions that can be retrieved by AF4.

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1. Introduction

In many domains of chemistry and life sciences, unravelling the complex pathways of molecular associations ranging from biomolecular complexes to the formation of larger aggregates, nanoparticles, and other supra-molecular structures is vital to understand the properties and behaviour of molecules and systems. Knowledge of associations is particularly relevant in the context of rational design of molecules with desired properties [1]. For example, in-depth characterization of the aggregate structures can reveal new targets for drug development. Protein aggregation or oligomerization is a highly complex process resulting in the formation of aggregates of various structures and morphologies. Aggregation may greatly affect protein properties. For example, many aggregates are cytotoxic and aggregates have been identified as the cause of several human neurodegenerative diseases [2]. Assemblies of polymers forming nanostructures have found a wide variety of applications in the area of nanotechnology, opening up new paths to develop drug-delivery systems and nanomaterials for biomedical applications [3]. Fabrication of such structures requires manipulation of the molecular interactions in an accurate and controlled manner. Additionally, detailed characterization of the resulted structures can reveal ways to obtain a desired assembly. In medicine a foreign substance introduced into a biological system should show as little interaction as possible, except with its intended target. This requires a thorough understanding of its physicochemical properties and a detailed risk assessment of potential consequences of unspecific binding (e.g. formation of protein corona) [4]. To date monitoring of molecular associations and assessment of the structure and architecture of supra-molecular conglomerates, especially in complex samples, poses unmet analytical challenges and new, complementary technologies are needed. This not only concerns the currently often limited selectivity, resolution and sensitivity in macromolecular separation and detection methodologies, but also the challenge of conserving structural integrity, molecular conformation and/or activity during analysis [5,6].

Field-flow fractionation (FFF) is an extremely flexible and versatile family of separation techniques, invented and patented in 1966 by J. Calvin Giddings [7]. FFF has advanced considerably during the last decades and has become a well-established separation technique for the separation and characterization of complex macromolecules [8–12], colloidal [13,14] and particulate systems [13,15–17]. The various FFF techniques share the same separation principle, but they employ different external separation fields. Samples are fractionated inside an open confined channel with a continuous flow, under the influence of an external field (e.g. flow, gravitational/centrifugal, thermal, electric, magnetic, electric, acoustic). The separation mechanisms of the FFF techniques have been extensively covered in literature and will not be discussed in this review [8,9,18–20]. Commercially available types of FFF instruments include (asymmetrical) flow FFF (AF4), hollow-fiberflow FFF (HF5), electrical AF4 (EAF4), thermal FFF (TF3 or ThFFF), centrifugal/sedimentation FFF (CF3 or SdFFF) and split flow thin cell fractionation (SPLITT). Of these, AF4 is undoubtedly the most-developed and most-widely used of the FFF techniques. Innovations in the instrumentation and a more comprehensive understanding of the theoretical principles of the FFF technique have assisted to attract new users and applications. In AF4, separation of the various species occurs in a ribbon-like open channel that consists of a non-permeable upper wall and a semipermeable accumulation wall at the bottom which is made of an ultrafiltration membrane and a solid frit. The underlying force to separate the various analytes, the so called cross-flow, is a flow field directed perpendicular to the laminar flow of the carrier liquid.

Some of the indisputable advantages of the FFF techniques, especially in comparison with chromatographic techniques, are derived from the separation mechanism and the absence of a stationary phase or packing material. Because of the use of an open channel a wider size range (from 10^2 Da or even lower to approximately 10^5 Da; particle diameter from 1 nm to 1 μm) [21–24] and more polydisperse samples can be analysed while avoiding filtering effects. The shear forces often experienced by the larger molecules passing through the narrow channels of a packed bed as well as the unwanted interactions between the analyte and the packing material are also eliminated. This is important, as studies have shown that considerable degradation, elongation, or denaturation of analytes may occur as a result of these forces and interactions [25–31]. FFF is considered a "gentle" separation technique, i.e. it exerts very little stress on the analyte molecules or particles. Another benefit of FFF is the great freedom to tailor the carrier-liquid composition to the needs of the application, without ill effects on the separation. A wide range of solvents or buffers can be used. Careful optimization of the carrier liquid composition in FFF is crucial to avoid undesired sample-membrane interactions and resulting sample loss. Complex, broadly dispersed multicomponent samples can be analysed in a quasi-native state without extensive sample preparation. Although mildly, evidently the sample may to some extent be affected by an FFF experiment. In AF4 the sample goes through a "relaxation" stage before being diluted in the carrier liquid during fractionation. FFF experiments cannot be carried out in situ. FFF is increasingly used as a mature method for size determination and fractionation of ultra-high-molecular-weight polymers, particles, labile biomacromolecules, and agglomerates. The above advantages make FFF, and especially AF4, eminently suitable to study molecular interactions between various systems, such as interactions between (bio-)macromolecules, association of proteins, self-assembling of polymers, nanoparticle-protein interactions, etc.

Finally, AF4 is (by definition) a fractionation method, which implies that fractions of the sample can be subjected to many additional characterization methods. FFF can be coupled to various detectors enabling determination of key analyte parameters, such as molar mass, size, density, diffusivity, surface charge, aggregation, conformation, shape. Standard concentration-dependent detectors include UV/Vis absorption, differential-refractive-index (dRI), and fluorescence (FLD) detectors [32–34]. For size-determination,
multi-angle-light-scattering (MALS) and dynamic-light-scattering (DLS) detectors are commonly employed. While DLS allows the determination of the diffusion coefficient through the hydrodynamic radius ($R_d$), MALS estimates the molar mass and the root-mean-square radius ($R_m$) of analyte molecules or particles [35–38]. If additional characterization is required, other detection techniques can be coupled online or offline to FFF. This includes inductively coupled plasma mass spectrometry (ICP-MS) [39] and other MS techniques (e.g., matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) [40] and electrospray (ESI-MS) [41,42], viscometry (Visc) [43,44], infrared spectroscopy (IR) [26], nuclear-magnetic-resonance spectroscopy (NMR) [45], electron microscopy [46,47], and, more recently, Raman microscopy [48] and surface plasmon resonance (SPR) [49] [8,50,51].

Although AF4 has found ample use in the fields of nanotechnology [15], nanomedicine and biotechnology [16,19,51,52] and also in the area of food [53], (bio-)polymer [8,14,18,54,55], and environmental sciences [13], the number of applications can still not compete with that of chromatographic techniques. The cost of the specialized equipment and the need for trained personnel are significant inhibiting factors, but, perhaps more importantly, the potential of the technique is still not fully realized. FFF, and especially AF4, are primarily used as sizing techniques, as may be seen from the applications documented in recent review articles. Wernert et al. [52], Zattioni et al. [16] and later Contado [15] focused on the use of AF4 for the size characterization of nanoparticles and, to a lesser extent, on its use to study the stability and release-properties of drug-delivery particles. The main advantages and limitations of AF4 for the characterization of several types of nanoparticles were addressed in these reviews. Malik et al. [8] provided an extensive report on the applications of AF4 and TF3 for polymeric materials and polymer nanocomposites, highlighting the advantages of the technique over size-exclusion chromatography (SEC) for fractionation and size determination of ultra-high-molar-mass and branched polymers. Zhang et al. [51] focused on the advantages of gentle FFF separations for the characterization of protein complexes and sub-cellular particles. In a number of publications more-specific guidelines and detailed protocols were reported for optimized FFF separation of specific analytes, such as viruses and virus-like particles [56] and nanoparticles [57]. The recent review of Quattrini et al. [50] more-thoroughly discussed the suitability of AF4 to study interactions between polymeric nanocarriers and biomolecules and to monitor drug-loading and release effects. Nonetheless, clearer documentation on the information that can be retrieved from FFF techniques, aside from the size and size distribution, will greatly contribute to the advancement of FFF techniques.

This review article is intended to provide a detailed overview of the developments in AF4 during the last 15 years, specifically for studies involving interactions and association of (bio-)macromolecules, such as proteins and polymers, DNA and nanoparticles. Our objective is to highlight how the information (e.g., size, shape, density, conformation, metal composition) that can be obtained using FFF techniques, especially when coupled to various detectors, can be used to provide insights in the molecular interactions while characterizing the resulting structures. Based on the FFF information, estimation of relevant interaction parameters, such as the dissociation constants, the aggregation kinetics and the binding stoichiometry, can be obtained. The possibilities, but also the practical limitations of FFF, will be outlined. Additionally, a critical comparison of AF4 with other techniques (e.g., SEC, CE) commonly used to study interactions, will be reported. While the emphasis is mostly on AF4, which is by far the most widely used FFF technique, noteworthy mentions of other FFF techniques, such as thermal field-flow fraction (TF3) and centrifugal/sedimentation field-flow fractionation (CF3) will also be included. We will first discuss interactions involving proteins, followed by discussions on interactions involving polymers and involving DNA. A large part of this review is devoted to the use of FFF for studying nanoparticles, a field that has seen strongly growing activity, mostly due to recent developments in drug delivery systems.

2. Interactions involving proteins

Studying interactions between proteins is vital for understanding biological functions and processes, which in turn is essential, for example, for developing effective protein therapeutics. The methodologies and analytical approaches to study protein–protein interactions are numerous, involving in vitro approaches, such as co-immunoprecipitation, X-ray crystallography, surface plasmon resonance (SPR), and chromatography. Usually a combination of techniques is necessary to confirm and characterize protein interactions [58,59]. One approach to understand the result of the interaction between two or more proteins is to monitor and characterize the formed structures after their binding. Weak and transient non-covalent protein complexes and assemblies are difficult to preserve during the analysis and many conventional approaches and techniques may be deficient [58,60–62]. FFF is an interesting technique to study weak protein interactions, due to its gentle and sorbent-free separation mechanism. Weak binding interactions with receptors or ligands [63,64], labile dynamic equilibria between various oligomeric protein forms [31] and the stability of protein aggregates have been studied with AF4 [65]. In Table 1 examples of studies using AF4 and HF5 to probe protein–protein interactions are summarized. Information on important separation parameters is included, and additional characterization techniques used by the authors are listed.

In this context, AF4 has been successfully employed to study the binding strength and stoichiometry of immunoglobulin G (IgG) to the neonatal Fc receptor (FcRn) and Fcγ receptor (FcγR) [64]. Monitoring the complexation between full-length IgG and its receptor (IgG/FcRn) with SEC showed no binding between the antibody and its receptor, possibly because of shear-induced dissociation of the weakly bound complex during the analysis. In contrast, AF4 using phosphate-buffered saline allowed the identification of the low-affinity IgG-receptor complexes (IgG-FcRn) and further separated the unbound receptor FcRn. The binding stoichiometry between the IgG and Fc-receptor was determined to be 2:1 using a calibration curve (constructed using multiple protein standards with various molecular masses) and the AF4 elution time of the FcRn, IgG monomer and dimer. AF4 data were used to provide an estimate of the binding affinity in terms of the equilibrium dissociation constant ($K_d = 3.74 \mu M$) between IgG and the FcRn receptor. FcRn at various concentrations was incubated with IgG at a fixed concentration and the mixture was further analysed by AF4. Based on the obtained peak areas of the unbound IgG, the fractional occupancy of the receptor was calculated. This latter property was plotted against the FcRn concentration. The best fits of this plot for both two equivalent binding sites and two non-equivalent binding sites models resulted in the same $K_d$ value, which was comparable with previously reported results obtained with other techniques, such as surface-plasmon resonance (SPR), gel-filtration chromatography and isothermal titration calorimetry (ITC).

AF4 was also proven suitable to monitor interactions between multicomponent complexes [53,64,66]. The interactions and associations between immunoglobulin G (IgG), the neonatal Fc receptor (FcRn), and human serum albumin (HSA) were studied. These types of three-component systems have greater physiological relevance than the widely studied two-component systems of IgG and FcRn. The AF4 results revealed an additional possible binding site of IgG.
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and FcRn, and a predominant HSA/FcRn/IgG/FcRn complex. This was not in agreement with the information obtained by SPR and immunoblotting according to which HSA/FcRn/IgG/FcRn/HSA had to be the main species formed. The experimental conditions and more specifically the concentration of each molecule used for the complex formation, was suspected to be crucial for the stoichiometry of the predominant species in solution and could explain the discrepancy between the techniques [64]. AF4 appeared to provide more-representative in-solution information in comparison with SPR or other techniques that required immobilization of the proteins on a specific matrix. Additionally, the ability of AF4 to separate analytes across a wider size range proved of great added value, as the associated complexes were in the 500–800 kDa range.

Combining AF4 with DLS and MALD considerably increases the quality and quantity of the obtained information. The size and molar mass of complexes typically do not suffice to comprehend interactions. From AF4–MALD information on the radius of gyration ($R_g$) and the molar mass distributions (MMD) can be extracted, while the hydrodynamic radius ($R_h$) can be obtained from AF4–DLS. The ratio $R_g/R_h$ provide an indication of the molecular shape in solution. Additionally, the apparent density ($d_{app}$) can be calculated from molar mass and $R_g$ distributions to understand variations in molecular shapes. Monitoring of the shape and density of the proteins and their aggregates over time or under stress conditions can provide valuable information on structure-function relationships. The cross-linking of caseins with transglutaminase (mTGase) was investigated with AF4–MALD–DLS [67]. Upon extensive incubation of the casein proteins with mTGase, a change in the shape of the cross-linked casein aggregates was revealed. After 1 h, the particles appeared like a micro-gel, whereas prolonged incubation led first to branching, and after 24 h to the formation of dense and spherical casein aggregates. In comparison, the non-cross-linked aggregates appeared as less dense, elongated structures. Better understanding of these structures may assist to the production of yoghurt-based products with improved properties.

Protein-protein interactions often have to be studied in extremely complex matrices. Prime examples of these are egg yolk [68], milk [69], and whole blood [69]. Being able to analyse proteins in such media with AF4, often with minimal sample preparation (no filtration, centrifugation or extraction), is facilitating a better understanding of their behaviour, as the formed complexes and/or aggregated species are more likely to be preserved. Despite documented applications of AF4 for the characterization of complex binding systems, there have been concerns that several steps during the separation process may affect labile or weakly bound protein complexes and aggregated species. The impact of the focusing/relaxation process, the carrier liquid composition and the dilution on the aggregate stability have been evaluated [31,65,70,71]. The composition of the carrier liquid is very important not only because it can induce the protein-protein interactions but also because optimal carrier liquid composition is necessary to avoid unwanted protein-membrane interactions [71,72]. The pH, ionic strength, salts and additives (e.g. metal ions, surfactants) have to be carefully selected when analysing proteins with AF4. The dilution in the AF4 channel was calculated on average to be 10-fold less than in SEC and the greater dilution was found to occur at the channel outlet. Therefore, according to Bria et al. [63], depending on the rate of dissociation of the aggregates, AF4 retention theory may provide more-accurate size information than online light-scattering detectors. The focusing process in AF4 is one of the primary steps suspected to influence aggregation and oligomerization, as it concentrates the sample close to the accumulation wall. Presumably the process may also lead to interactions with the membrane, especially if long focusing times are used. However, prolonged focusing times (up to 13 min) showed no significant change in the weight-average molecular weight ($M_w$) of weakly bound anti-streptavidin (anti-SA) IgG aggregates, and no change in the relative amounts of the monomer and aggregated species [65]. The influence of the focusing process on the oligomerization (“tetramerization”) of the enzyme β-D-galactosidase was evaluated with AF4 and frit-inlet AF4 (FI–AF4) [31]. In FI–AF4, hydrodynamic relaxation can be achieved through a “stop-less” injection, avoiding undesirable effects of the focusing process. Association of the dimeric species in the presence of metal ions was observed by AF4 and FI–AF4. The in-solution oligomerization was verified by batch-mode DLS, confirming that the focusing process is not the cause of the protein oligomerization. The impact of these parameters and processes should be considered when applying AF4 for separation of labile protein complexes and transient aggregate populations.

HF5 is a specific variant of AF4 that has shown promising results for the separation and quantification of proteins and protein aggregates. In HF5 the channel is replaced by a semipermeable hollow fibre, with the cross-flow being exerted on the entire circumference. Although the separation principle of HF5 is fundamentally the same as that of AF4, the implementation has some advantages over the conventional AF4 set-up. In HF5 the ratio of membrane area to channel volume is more favourable. This should lead to less dilution during the analysis. The small channel size is also advantageous in sample-volume limited situations [73]. The lower flow rates encountered in HF5 enabled coupling with additional instrumentation, such as MALDI/TOFMS and ESI/TOFMS [74]. However, at present HF5 does not yet offer the same flexibility regarding channel dimensions, membrane materials, and membrane pore-size as AF4. The current practical limitations of HF5 may be one reason for the popularity of AF4 miniaturization [42,75–78]. Moon et al. demonstrated the coupling of a miniaturized AF4 chip with ESI–MS for top-down analysis of size-fractionated proteins [42] and for lipid analysis of plasma lipoproteins [76], and with ICP–MS for the characterization of metalloproteins [78].

2.1. Protein-aggregation kinetics

AF4 has been used to investigate the kinetics of protein aggregation processes and the effects of external factors, such as centrifugation [79], temperature [80], presence of additives [81,82], on the protein structure and aggregate formation. Based on the information that can be obtained by AF4, such as the relative quantity, size and shape of the native and aggregated protein species, kinetic models and the mechanisms leading the aggregation can be determined. Bria et al. [79] demonstrated the use of AF4 for the characterization of submicron (0.1–1 μm) aggregates of a heat-stressed anti-streptavidin (anti-SA) immunoglobulin G antibody (IgG). The influence on the aggregation kinetics of the high molar mass submicron aggregates and larger materials was investigated in the presence of these species and after their removal by centrifugation. They elucidated the aggregation kinetics using the Lumry–Eyring nucleated polymerization (LENP) model, which required results from AF4 experiments, such as the monomer fraction (m) and molar mass of the monomer (M_monom) and aggregated species (Magg). The monomer fraction (m) is determined based on the peak area of the monomer of the stressed sample relative to the peak area of the monomer in the unstressed sample. The kinetic data obtained for centrifuged and uncentrifuged heat-stressed anti-SA IgG samples were fitted to the model. It was proven that the centrifuged heat-stressed sample adhered to the same kinetic mechanism as the uncentrifuged sample (Fig. 1). A potential mechanism involved slow nucleation and aggregate-aggregate condensation.
Borzova et al. [80–82] proposed two additional methodologies for elucidating the aggregation kinetics of bovine serum albumin (BSA) when treated at high temperatures (from 60 °C up to 80 °C) and in the presence of additives (dithiothreitol and arginine derivatives) based on AF4 measurements. Valuable information on the initial stages of thermal aggregation was obtained by plotting the amount of aggregated protein ($\gamma_{agg} = 1 - \gamma_{non-agg}$) against the fraction of the denatured protein ($\gamma_{den} = 1 - \gamma_{nat}$). Based on the relationship between the aggregated and denatured forms it was revealed that two unfolded species with different aggregation tendencies were formed upon thermal stress. Additionally, the hydrodynamic radius of the protein aggregates ($R_h$) estimated by DLS was plotted against the fraction of the aggregated protein ($\gamma_{agg}$), estimated from the UV absorbance peak area. According to the authors, the shape of the extracted $R_h$ vs $\gamma_{agg}$ plot provides insights in possible changes in the aggregation pathways when varying environmental conditions [82]. Based on these plots, BSA upon thermal stress was found to form aggregate assemblies, which were detectable in the presence of arginine and its derivatives.

A very interesting application of sedimentation FFF (SdFFF) to study the aggregation behaviour of protein emulsions has been reported by Kenta et al. [83]. In emulsions formulated and stabilized with milk proteins, competitive adsorption between the protein fractions occurs at the oil-water interface. Being able to control the first protein adsorbed at the interface is of great importance for the food industry. This protein is more likely to remain in the emulsion during storage or further processing, enhancing the stability of the emulsion. Investigation of the adsorption phenomena in the presence of complex mixtures of milk proteins, in which different protein fractions coexist during the emulsification process, can shed light on the factors influencing the adsorption. The complexity of these phenomena is enormous, as non-ionic surfactants can also effectively displace the milk proteins from the emulsion interface. Kenta et al. used SdFFF to monitor changes in the particle-size distribution of emulsions (weight average diameter from approximately 300 up to 500 nm) prepared with sodium caseinate (SC and heated at (30.5 and 80.0 °C) for a 70 h period. Emulsions exhibited an initial increase in the droplet size upon heat treatment, followed by a decrease to approximately the initial size. The experimental rate constants of the initial steps of aggregation obtained with SdFFF revealed the formation of an intermediate complex of oil droplets, followed by the formation of stable aggregates. The rate of oil-droplet aggregation was found to be temperature dependent. A number of physicochemical quantities that affect the stability of the oil-in-water emulsions were also determined.

3. Interactions involving polymers

3.1. Polymer-association interactions

The field of polymer synthesis has advanced towards the preparation of larger, more-complex and better-defined polymer architectures, including supramolecular assemblies [88]. Comprehensive characterization of such complex structures is critical for developing new materials and improving existing ones. Determination of the distributions of molar mass (MMD), chemical composition, functionality, and molecular topology (including the degree of branching) is essential to establish structure-property correlations. The analytical techniques used for the characterization of new polymeric materials are being challenged by the increased sample complexity. Spectroscopic techniques, such as NMR and Fourier-transformed infrared spectroscopy (FTIR) provide only average values and do not provide insights on polymer distributions, while light-scattering techniques (such as DLS) only
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<tr>
<td>Lysine-dendronized maleimide with maltose shell</td>
<td>AF4-MALS-dRI 350 µm spacer RC membrane (10 kDa MWCO) F&lt;sub&gt;c&lt;/sub&gt;: 1.5 mL min&lt;sup&gt;-1&lt;/sup&gt; F&lt;sub&gt;out&lt;/sub&gt;: 1.0 mL min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>0.02% Na&lt;sub&gt;N&lt;/sub&gt;</td>
<td>DLS, NMR, SEC, TEM</td>
<td>• AF4-MALS to study the self-assembly of dendrons that possess extremely dense branching, including effects of generation number, pH, temperature, and concentration on aggregation behaviour</td>
<td>[21]</td>
</tr>
<tr>
<td>Polyion complexes (PICs)</td>
<td>FI-SyFFF-UV/Vis-dRI 250 µm spacer RC membrane (10 kDa MWCO) F&lt;sub&gt;c&lt;/sub&gt;: 4.0 mL min&lt;sup&gt;-1&lt;/sup&gt; F&lt;sub&gt;out&lt;/sub&gt;: 0.6 mL min&lt;sup&gt;-1&lt;/sup&gt; FI-AF4-UV/Vis-DLS-MALS-dRI 250/350 µm spacer F&lt;sub&gt;c&lt;/sub&gt;: 2.0 5 mL min&lt;sup&gt;-1&lt;/sup&gt; F&lt;sub&gt;out&lt;/sub&gt;: 1.0 mL min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>0.02% Na&lt;sub&gt;N&lt;/sub&gt;</td>
<td>DLS, NMR, SEC, TEM</td>
<td>• SEC claimed not to be an option for these ultra-high-molecular weight structures (shear forces, mobile-phase incompatibility)</td>
<td>[98]</td>
</tr>
<tr>
<td>PVA-g-PMMA amphiphilic copolymer</td>
<td>AF4-MALS-dRI 490 µm spacer RC membrane (5 kDa MWCO) F&lt;sub&gt;c&lt;/sub&gt;: 3.0 mL min&lt;sup&gt;-1&lt;/sup&gt; F&lt;sub&gt;out&lt;/sub&gt;: 0.5 mL min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>Deionized water</td>
<td>DLS, ELS, SANS, SLS, TEM</td>
<td>• AF4-MALS to investigate the self-assembly and complex nanostructure of an amphiphilic PVA-g-PMMA colloidal system</td>
<td>[108]</td>
</tr>
</tbody>
</table>

provide minimal information on distributions. Understanding and monitoring of the distributions may help to improve the properties of polymers and to predict their stability and functionality.

Size-based separation techniques are used to acquire information on molecular-size or MMDs [89]. SEC is the most-popular size-based separation technique. However, it has certain limitations when it comes to the analysis of ultra-high-molar-mass samples of, for example, block copolymers, polyelectrolytes, or polymer self-assemblies. Filtering effects, shear degradation, and adsorption on the stationary-phase surface have been reported to lead to inaccurate MMDs [25–30]. Additionally, linear and branched polymers of different molar masses may co-elute, due to similar hydrodynamic volumes [25,26]. FFF has found ample use for the characterization of synthetic and natural polymers, because it can overcome the above limitations of SEC [8,89]. The use of FFF for the characterization of polymers, polyolefins and polymeric nanocomposites has been discussed in detail in reviews of Messaud et al. [90] and Malik et al. [8]. The present review, focuses on FFF-based studies into the association of polymers. In Table 2 examples of studies using AF4 and TF3 to monitor the association of polymers and the respective conditions used are summarized.

FFF (especially AF4 and TF3) has been used to study the convoluted mechanisms of association of polymers (self-aggregation or self-assembly) that result in the formation of nano-to micrometer sized assemblies of nano-to micrometer sizes. The terms association or self-assembly (that result in the formation of supramolecular assemblies of nano-to micrometer sizes) have been discussed in detail in reviews of Messaud et al. [90] and Malik et al. [8]. The present review, focuses on FFF-based studies into the association of polymers. In Table 2 examples of studies using AF4 and TF3 to monitor the association of polymers and the respective conditions used are summarized.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>FFF Conditions</th>
<th>Eluent</th>
<th>Additional techniques used in the study</th>
<th>FFF highlights</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charged block copolymer of anionic PMMA-b-PMMA, Cationic P2VP-PS and P4VP-PS</td>
<td>TF3-UV/Vis-MALS-DRIL</td>
<td>—</td>
<td>—</td>
<td>• TF3 coupled to multidetector system to characterize charged self-assemblies [93] • TF3 to separate charged micelles based on corona composition</td>
<td>—</td>
</tr>
<tr>
<td>Amphiphilic PS–PEO block copolymers PS305–PEO841, PS981–PEO773 (subscript indicates the degree of polymerization for each block)</td>
<td>TF3-UV/Vis-MALS-DRIL</td>
<td>1 mM LiBr</td>
<td>DLS, STEM imaging</td>
<td>• TF3 to fractionate complex morphologies, such as jellyfish and worm-like [105]</td>
<td>—</td>
</tr>
<tr>
<td>1,4 and 1,2 PB–PS micelles</td>
<td>TF3-UV/Vis-MALS-DRIL DLS</td>
<td>Heptane</td>
<td>—</td>
<td>• TF3 coupled to multidetector system to monitor the dynamics and formation of mixed micelles by “unimer” exchange between 1,4 and 1,2 PB-PS micelles • TF3 to fractionate PB-PS micelles based on the corona composition</td>
<td>—</td>
</tr>
<tr>
<td>1,4 and 1,2 PB–PS micelles</td>
<td>TF3-UV/Vis-MALS-DRIL DLS</td>
<td>N,N-dimethylacetamide (DMA)</td>
<td>1H NMR, TEM</td>
<td>• TF3 to investigate the influence of core microstructure on the solution properties of the block copolymer self-assemblies • Differences in morphology and chain arrangement were shown to influence the separation of the self-assemblies • The impact of the temperature gradient (ΔT) on the self-assembly properties was monitored with DLS and NMR</td>
<td>—</td>
</tr>
<tr>
<td>PS-b-PMMA block copolymers</td>
<td>TF3 X SEC-UV Vis-ELSD</td>
<td>Tetrahydrofuran (THF)</td>
<td>DLS</td>
<td>• Hyphenation of TF3 (1st dimension- separation on size and chemical composition) and SEC (2nd dimension- separation on size/hydrodynamic volume) explored to fractionate blends of homopolymers, homo- and copolymers, and copolymers of different compositions</td>
<td>—</td>
</tr>
</tbody>
</table>

Polystyrene (PS), polyacrylic acid (PAA), poly(ethylene glycol acrylate) (PEGMA), poly(N-isopropylacrylamide) (NIPAM), poly(γ-benzyl-L-glutamate) (PBLG), poly(ethylene oxide)-block-poly(furfuryl glycicyl ether)-block-poly(allyl glycidyl ether) (PEO-b-PFG-b-PAGE), poly(ethyleneoxide-b-c-caprolactone) (PEO-b-PCL), poly(vinyl alcohol)-graft-poly(methyl methacrylate) (PVA-g-PMMA), poly(methacrylic acid)-b-poly(methyl methacrylate) PMMA-b-PMMA, poly(2-vinyl pyridine)-b-polystyrene (P2VP-PS) and poly(4-vinyl pyridine)-b-polystyrene (P4VP-PS), polystyrene-poly(ethylene oxide) PS-PEO, polystyrene-poly(methyl methacrylate) block copolymers (PS-b-PMMA).
present and on the non-covalent interactions between these [3].
The impact of a multitude of parameters on these structures, such as the polymer composition [91,92], temperature [93], solvent [94] and pH [21,38] has been studied with FFF. Hyphenation of FFF with various detectors can yield comprehensive information of the architecture [21,95] and morphology [96,97] of the resulting structures.

The characterization of polymers with molecular weights above 1 MDa and investigation of their supramolecular assemblies has been demonstrated with AF4. Boye et al. [21] synthesized a series of large maltose-modified and lysine-dendronized maleimide co-polymers. Lysine dendrons that were formed in different numbers of repeated branching cycles (so called “generations”) were used. Several analytical and computational tools (NMR, SEC, transmission electron microscopy (TEM), atomic force microscopy (AFM), molecular modelling), including AF4-MALS and DLS, were used to study the influence of various parameters, viz. i) generation number, ii) concentration of the samples, iii) temperature and iv) pH, on the intermolecular interactions of these structures. The influence of increasing the number of generations of dendrons on the formation rod-, coil-, and worm-like molecular structures with controlled aggregation was explored. The degree of aggregation (or aggregation number: \( M_w/\langle M_w\rangle \)) was quantified based on the weight-average molar mass of the aggregates (\( M_w \)) and the molar mass of the single macromolecule. \( M_w \) values were determined from the AF4-MALS data. To understand the type of aggregates formed, the apparent density (\( d_{agg} \)) and shape factor (\( R_g/R_h \)) were also determined. The aggregation was shown to depend strongly on the shape of the macromolecules and the solution conditions (pH, concentration). An increase in polymer concentration led to a slight increase in size, whereas temperature (in the range 25–90 °C) was not found to affect aggregation. This type of information can assist in the design and fabrication of structures with specific functions, which may be particularly interesting for medical applications.

The impact of the architectural complexity of five different amphiphilic polymers on micelle formation was studied using AF4-MALS, batch-mode DLS and TEM by Lonsdale et al. [95]. A so-called 3-miktoarm star block copolymer and a more-complex dendrimer-like block copolymer consisting of polyacrylic acid (PAA) and polystyrene (PSTY) were used to form micelle structures. The size, shape, and morphology of these structures were monitored. AF4-MALS was used to determine the average molecular weight of the formed micelles and the degree of aggregation (\( Z_{agg} \)), which represents the number of “unimers” per micelle. An increased architectural complexity of the polymers used for the micelle formation led to more-spherical core-shell aggregates in water with an increased hydrodynamic diameter (estimated by DLS), average radius of the core in the dry state (estimated by TEM) and aggregation number (estimated by AF4-MALS). It was concluded that the self-assembly process was driven by entropic factors due to the formation of loops in the core, which reduced the repulsive forces and increased the number of unimers and the size of the formed micelles.

Micellar structures of various morphologies formed by a complex self-assembled ABC triblock terpolymer, i.e. poly(ethylene oxide)-block-poly(furfuryl glycidyl ether)-block-poly(allyl glycidyl ether) (PEO-b-PFGE-b-PAGE), were synthesized and then characterized using AF4-MALS, batch mode DLS and TEM [38]. The study highlighted the complementarity of these techniques, which was necessary to investigate the variety of morphologies that can be formed. Upon systematic optimization of the AF4 separation conditions, mainly the cross-flow rate (0.5–1.5 mL min\(^{-1}\)) and the focusing time (3–11 min), certain limitations were described regarding interactions of the analyte with the membrane, which influenced the retention behaviour of the triblock terpolymers. The elution behaviour of the triblock terpolymers was investigated at various separation conditions of ionic strength (NaCl concentration from 0 to 150 mM), pH (4.0–9.9) and presence of metal ions (Fe\(^{3+}\)). No significant influence of the pH and ionic strength was reported, but the addition of Fe\(^{3+}\) induced corona cross-linking and formation of particles, the size of which depended on the amount of Fe\(^{3+}\) in the solution. Depending on the type of functionalization of the ABC triblock terpolymer, either with carboxy groups or a fluorocarbon chain introduced in the PAGE segment of the polymers, spherical or worm-like micellar structures were formed. The estimated \( R_g \) and \( R_h \) and shape factor (\( R_g/R_h \)) values obtained by AF4-MALS-DLS allowed distinguishing between the two morphologies. Cryo-TEM revealed the presence of additional small disc-shaped structures, for which reliable \( R_g \) and \( R_h \) values could not be obtained [38].

In a recent study, AF4 was used to monitor the self-assembly of triblock poly(BLG-b-NIPAM-b-PEGA) copolymers to form anisotropic polymeric nanoparticles. Upon nanoprecipitation, coexistence of spherical and elongated particles was revealed (Fig. 2) [97]. The poly(ethylene glycol acrylate) (PEGA) outer block provided surface hydrophilicity and colloidal stability, while the thermo-responsive property was induced due to the poly (N-isopropylacrylamide), poly (NIPAM), block which alters size and hydrophobicity through temperature variations. Overlaying the radius of gyration (\( R_g \)) estimated by MALS and the LS signal, two regions could be distinguished. In the earlier eluting particles (Fig. 2, region I) MALS data could be fitted using a sphere model, whereas in the later eluting particles (Fig. 2, region II) the same fit
could not be applied, indicating the presence of non-spherical particles. Plotsting the shape factor \((R_g/R_h)\) along the elution the coexistence of spherical \((R_g/R_h \approx 0.77)\) and more elongated \((R_g/R_h > 0.77)\) particles was evident. Additional information on the shape of the formed particles was obtained by TEM and was in good agreement with the AF4 results [97].

To obtain a comprehensive picture of the self-assembling process and the resulting products, it is necessary to preserve the structural integrity of the molecules during analysis. This is a major analytical challenge especially when dealing with complexes and assemblies prone to dissociation, such as shear sensitive polyelectrolyte complex micelles (PICs) [98]. In such a case, the FFF process must be carefully considered, particularly the sample relaxation and focusing steps. Frit-inlet FFF (FI-FFF) was proven to preserve the structure of the PICs and to identify changes in their composition as a function of NaCl concentration.

It has been argued that TF3 may become a prominent FFF technique for the characterization of polymers and polymer self-assemblies [93,99–101]. A comprehensive description of the principles and theoretical details of TF3 can be found elsewhere [102]. In TF3 the separation force is established by applying a temperature gradient across the channel. The top wall of the channel is heated, and the bottom wall is cooled. Commonly, analysed polymers are driven to the cold wall (thermal diffusion coefficient, \(D_T\)). \(D_T\) is influenced by the polymer-solvent interactions and is strongly related to the chemical composition of the polymer. Consequently, in TF3 analytes are separated based on their molar mass, but also according to their chemical composition, yielding a confounded picture that may be hard to interpret. Thermal diffusion is also highly variable and difficult to predict. TF3 is mainly used for characterizing polymers in organic carrier liquids, because \(D_T\) values in aqueous systems are negligible, with a notable exception of micellar systems [93] and colloid polystyrene beads in aqueous dispersion [103].

In principle, all detectors used for AF4 are also applicable also for TF3. TF3 was coupled to five detectors, namely UV/Vis absorption, dRI, MALS, DLS and Visc. This platform requires careful interpretation of the data, but has been claimed to provide simultaneous quantitative information on a number of important molecular parameters, including molar mass, chemical composition, molecular topology, intrinsic viscosity, and thermal diffusion coefficient [101]. TF3 has been applied for the separation and characterization of i) charged block copolymers [93], ii) block copolymers with varying core microstructure [99], iii) thermo-responsive star polymers [100], and iv) polymers of varying complexity and shape using the aforementioned pentadetection setup [101]. Fractionation based on the corona composition [104], core composition [99] and according to the morphology [105] have been achieved with TF3.

TF3 was used to monitor the structure and dynamics of polycatenated-polystyrene (PB-PS) micelles with various corona compositions. TF3-MALS-DLS provided information on the size, shape, polydispersity, and molar mass of the micelles. Hyphenation of TF3 with FTIR provided chemical-composition information about the corona of the micelles [104]. The PB isomer content (1,4- and 1,2-PB) was monitored during the elution, revealing the differences in micelle composition.

TF3 with aqueous carrier liquids was also demonstrated for fractionation of charged polymer self-assemblies [93]. Greyleng et al. [93] used aqueous TF3 to fractionate anionic and cationic micelles based on their corona composition and they studied the effects ionic strength and pH. The anionic micelles were formed by poly(methacrylic acid)-b-poly(methyl methacrylate) (PMAA-PMMA) block copolymers whereas the cationic micelles were formed by poly(2-vinyl pyridine)-b-polystyrene and poly(4-vinyl pyridine)-b-polystyrene (P2VP-PS or P4VP-PS) block copolymers. TF3 data revealed morphological changes of the self-assemblies at increased ionic strength, due to intermolecular changes in the corona. Additionally, separation of P2VP-PS and P4VP-PS micelles was achieved, demonstrating the potential of TF3 to separate charged self-assemblies based on their corona composition. Micelles prepared from amphiphilic block copolymers (ABCs) may exist in equilibrium of multiple morphologies, such as spherical, cylindrical, worm or vesicular. These were successfully separated with TF3 [105]. The self-assembling of two different polystyrene-poly(ethylene oxide) (PS-PEO) block copolymers was studied by Muza et al. [105] After characterization of the spherical PS-PEO micelles in acetonitrile (ACN), lithium bromide (LiBr; 1 mM) was added to the micelle solutions. The addition of the electrolyte induced morphological changes to the spherical micelles. Determination of the shape \((R_g/R_h)\) showed that PS985-PEO636 block copolymers, in which PS block was shorter than PEO block, assembled into worm-like micelles. Contrary, the PS981-PEO773 block polymer, in which the PS block is longer than the PEO block, formed cylindrical micelles. The fact that very complex morphologies existing in equilibria can be monitored and fractionated may broaden the application range of TF3.

Although TF3 offers unique features for the fractionation of complex polymers and polymer assemblies, a better understanding of the thermal diffusion mechanism is necessary to predict the retention characteristics and to interpret fractograms of unknown analytes [106,107].

### 3.2. Polymer-protein conjugates

Polymer-drug conjugate therapeutics form an important and rapidly growing group of drug-delivery systems [110]. Conjugation of bioactive molecules, including small molecules, peptides, proteins and aptamers, to polymeric carriers has been successfully implemented in clinical practice. Conjugation with polyethylene glycol (PEG) has shown numerous benefits, including enhanced drug solubility, controlled delivery, prolonged circulation, and reduced immunogenicity. Our focus is on the PEGylation of proteins (protein-PEG conjugates), which has found the greatest clinical success thus far.

Covalent bonds between PEG molecules and the protein biotherapeutics (enzymes, antibodies, etc.) lead to a significant increase in protein size. PEGylated proteins may be present in complex mixtures, consisting of unreacted protein molecules, products with a different degree of PEGylation, and possibly aggregates in solution. This complexity makes the generation, purification, and characterization of well-defined products very challenging.

Techniques such as SEC, SDS-PAGE, and CE have been used to obtain size distributions of polymer-protein conjugates [111–114]. Although SEC is the reference technique for determining size and polydispersity, the range of molecular weights that can be separated with SEC may be a limiting factor for the characterization of high-molecular weight conjugates and their aggregates. AF4 coupled to UV-MALS-dRI has proven a valuable alternative, suitable to monitor the results of the interactions between polymers and proteins [115–117].

The aggregation of a PEGylated protein (granulocyte-colony stimulating factor; PEG-G-CSF), stored in two different buffer solutions (10 mM acetate buffer with 5% sorbitol, pH 3.4 and 50 mM acetate buffer with 200 mM sodium chloride, pH 4.5), was investigated using both SEC and AF4 coupled to a multi-detection system (UV-MALS-RI) [117]. Both techniques confirmed the presence of a PEGylated protein conjugate and traces of unreacted protein, as well as high-molecular-weight (HMW) aggregates. Two SEC columns connected in series were necessary to provide comparable
resolution of the PEGylated protein conjugate and the aggregated species to that obtained by AF4. Furthermore, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to confirm the constituents present in the conjugate samples. The presence of PEGylated protein conjugate and unreacted protein was confirmed, whereas HMW aggregates could not be detected with MALDI-TOF-MS. The AF4-MALS results revealed that addition of 5% sorbitol to the storage buffer led to the formation of fewer aggregates. Monitoring of the aggregate formation is of great importance for the optimization of the synthesis conditions and the selection of proper storage conditions.

Although protein PEGylation is commonly used to shield therapeutic proteins, such as antibodies, enzymes, or growth factors, and to subsequently improve the protein stability in vivo, an associated decrease in biological activity is often observed. Repeated exposure to PEG-containing conjugates may potentially lead to the formation of anti-PEG antibodies in patients, which decrease the circulation time of the conjugates and increase immunogenicity [118,119]. Therefore, potential polymeric conjugate alternatives are being investigated [120–122]. Martin et al. [123] demonstrated a different approach to enhance the stability and bioavailability of IgG antibodies by noncovalent association with hydrophobically modified poly(acrylate) copolymers. AF4 was used to study the complex formation between poly(acrylates) and monoclonal IgG and to investigate the complex stability at elevated temperatures [116].

Two different methodologies were attempted for the conjugate formation revealed a minimum of two conjugated polymer chains per enzyme molecule for protease, whereas lipase and a-amylase were linked to three or eight polymer chains, respectively. mPEG-NHS showed the largest number of polymer chains attached to a-amylase and lipase. In combination with differential scanning microcalorimetry and UV assays, results confirmed that the synthesizing methods produced enzyme-polymer conjugates that were stable against detergents.

4. Interactions involving DNA

Tools to assess DNA–protein interactions and to provide information about the binding affinities are necessary for the discovery of new DNA sequences with affinity to various biomarkers [32,125]. Synthetic single-stranded DNA (ssDNA) or RNA molecules called aptamers are able to bind to specific targets, such as proteins, metabolites, and metal ions [126]. A deeper understanding of the nature of these interactions and the reasons that these may be interrupted is crucial for our understanding of numerous diseases, including cancers [127].

AF4 with fluorescence detection (FLD) was used for the isolation of an aptamer-protein complex and for the simultaneous monitoring of the binding between the fluorescently labelled aptamer and the target protein [32,125,128]. AF4 was exploited to separate the free and bound aptamers, as well as for in-channel incubation to form the aptamer-protein complex. Pre-incubated and not-incubated samples of aptamer and protein were analysed with three different focusing times (3, 6, 12 min) and the binding ratios were estimated from the peak areas of the aptamer-protein complex and the sum of the peak areas of the free and bound aptamer. Even the shortest focusing step was found adequate for in-channel incubation to form the stable aptamer–protein complex. The dissociation constant (Kd) between the aptamer and the binding target was estimated from the AF4-FLD fractogram, again based on peak areas. The Kd values found were not consistent with literature values (obtained with SPR). Both lower and higher values were obtained for different aptamer-protein complexes (aptamer-IgE and aptamer-streptavidin, respectively) [125]. Isolation of the DNA–protein complex and further investigation of the interactions often requires immobilization of the complex on a solid support [129]. Immobilization may impact the protein stability and introduce interferences from the support surface. Additionally, it is very difficult to conclude whether the interaction occurs with the target protein in its monomeric or aggregated form. Although the binding strengths observed for the immobilized (SPR, microarrays) and freely suspended proteins (AF4-FLD) appear to differ, AF4 was found to preserve the conformation of the protein and the protein–aptamer complex in solution. Additionally, the readily adjustable carrier-liquid composition allowed the use of high salt concentrations and the addition of divalent ions (Mg2+, Ca2+), producing an environment that can stabilize the folding of the aptamers [125,128]. Examples of studies involving AF4 used to investigate the interactions between DNA and target proteins are summarized in Table 3. The obstacles and possibilities related to the use of AF4 for studying these types of interactions are also included.

5. Interactions involving nanoparticles

5.1. Engineered nanoparticles

Nano-sized particles (engineered or natural nanoparticles, NPs; natural colloids) are widely used in consumer products (e.g. sunscreen, tooth paste, paint) to improve their properties [131,132]. However, when these nanoparticles are produced, consumed, and finally released into their intended environment, they are subjected to various structural and chemical alterations that can impact their toxicity. It is of great importance to determine whether nanoparticles are safe for the proposed application or whether they
impose health and/or environmental threats. Metal and metal-oxide NPs (e.g., comprised of Ag, TiO2, ZnO, CeO or Au), fullerenes and carbon-based nanomaterials (nanoplastics and carbon nanotubes) are currently the most-abundant engineered NPs [133–135]. The greatest analytical challenges regarding the analysis of such particles are i) detection and characterization of the particles present in complex media, ii) establishment of relevant conditions (e.g., ionic strength, pH, presence of natural organic matter) to predict their behaviour (aggregation/agglomeration, disassembly, surface modification changes, chemical transformations) and iii) monitoring interactions with other components present in the media they are released in. Monitoring changes in the size, size distribution and morphology can provide information to understand the stability, aggregation mechanisms, and interactions between nanoparticles. This may elucidate their fate when present in complex media, such as the environment [6], consumer goods [100] and nanoparticles. This may elucidate their fate when present in complex media and morphology can provide information to understand the monitoring interactions with other components present in the media.

Examples of studies investigating interactions between DNA and proteins using AF4.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>FFF Conditions</th>
<th>Eluent</th>
<th>Main results</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssDNA + Protein</td>
<td>FL-4F-UV/Vis 250 μm spacer RC membrane (10 kDa MWCO) Fc: 0.49 mL min⁻¹ Fm: 40 μL min⁻¹</td>
<td>100 mM Tris-HCl (pH 7.4)</td>
<td>• Successful attempt at measuring ssDNA and ssDNA bound to a protein using a miniaturized frit-inlet AF4 channel</td>
</tr>
<tr>
<td>Aptamer + IgE</td>
<td>AF4-UV/Vis-FLD 350 μm spacer RC membrane (10 kDa MWCO) Fc: 3.0 mL min⁻¹ Fm: 0.3 mL min⁻¹</td>
<td>PBS with MgCl₂</td>
<td>• AF4-UV/Vis-FLD used to determine the binding affinities between an aptamer and IgE</td>
</tr>
<tr>
<td>Proteins</td>
<td>AF4-UV/Vis-FLD PBS with 1.0 mM MgCl₂ (pH 7.4) and 20 mM Tris-HCl, 100 mM NaCl, 50 mM KCl, 20 mM MgCl₂, 10 mM CaCl₂ (pH 7.5)</td>
<td>• Based on the Kᵣ values derived from AF4 migration times folding state of aptamers was evaluated</td>
<td></td>
</tr>
<tr>
<td>DNA methyltransferase 1 (DNMT1)</td>
<td>AF4-UV/Vis-FLD 500 μm spacer RC membrane (10 kDa MWCO)</td>
<td>20 mM Tris, 50 mM NaCl, 50 mM dithiothreitol (DTT) and 10 mM MgCl₂ (pH 7.5)</td>
<td>• High salt concentrations, divalent cations (Mg²⁺, Ca²⁺) used as carrier liquid, allowing best binding environment to study interactive systems</td>
</tr>
<tr>
<td>DNA + Aptamer</td>
<td></td>
<td></td>
<td>• AF4 coupled to Systematic Evolution of Ligands by Exponential Enrichment (SELEX) to study interactions between aptamers and various protein targets</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Alternative methodology to study these interactions without protein immobilization</td>
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<td></td>
<td>• DNMT1 protein and ssDNA incubated in the AF4 channel; DNMT1-bound ssDNA complexes were separated from free ssDNA</td>
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</table>

Due to the sample complexity, motoring of changes in the composition of the NPs is necessary to understand the aggregation/agglomeration process and the particle-particle and particle-contaminant associations. Coupling AF4 to size-specific detectors (such as light scattering) and composition-sensitive detectors, such as (single-particle) ICP-MS or fluorescence detection, is vital to shed light on particle transformations and interactions in complex media [146–148].
marine water, whereas the free AgNPs had agglomerated and sedimented. Hence, organic matter present in the marine water was thought to stabilize AgNP and the coating formed was likely to change their interactions with surrounding living organisms.

Railean-Plugaru et al. [159] used AF4 in combination with MALS and DLS to prove that the surface of the NPs could affect their dispersion. Various complementary techniques, such as TEM, FTIR and MALDI-TOF-MS were used to provide additional compositional information. Synthesized silver nanocomposites (AgNCs) are used as antimicrobial agents, due to their effectiveness against numerous human pathogens. The size of AgNCs influences their physico-chemical properties, with significant impact on their toxicity and bioavailability. To gain accurate information on in-solution size and size distribution of the nanocomposites, AF4 coupled with UV, MALS, and DLS was employed using two mobile phases with different ionic strengths (deionized water and phosphate-buffered saline solution). All fractions obtained with AF4 and further analysed with TEM revealed the presence of differently sized particles, which contained one or more clusters of AgNCs with different organic surface coating. The hydrodynamic diameter and the radius of gyration of AgNCs obtained by AF4 (in solution) were slightly larger than the sizes obtained by TEM (in vacuum). The organic coating was found to strongly influence the hydrodynamic diameter and migration rate of the particles, an effect that could not be detected by TEM. As a result, the AF4 elution times were affected by both the final size of the particle in solution and the chemistry of the coating of the AgNCs. Considering that AgNCs show size-dependent cytotoxicity, the observed behaviour provides insights of great relevance for biomedical applications [159].

Recently, the impact of degrading micro- and nanoparticles on the environment and public health has attracted a great deal of attention. Although micro- and nanoparticles consist of biochemically inert polymeric materials, the likelihood of this debris being ingested by organisms, entering the food chain, and ultimately affecting human health is high [160]. Plastic particles can adsorb substances on their surface and transport these to marine and freshwater environments. AF4 was used to establish the presence of nanoplastics in environmental and food matrices and to study their aggregation/agglomeration behaviour [48,144,161,162]. Correia et al. [144] developed a methodology based on AF4-MALS-FLD for the characterization of polymeric nanoparticles, such as polystyrene (PS) and polyethylene (PE) present in a complex food matrix (fish tissue). Two types of sample preparation were used, viz. acid and enzymatic digestion. Results showed that enzymatic digestion allowed degradation of the fish matrix, without influencing the properties of PS nanoparticles, whereas acid digestion resulted in large agglomerates/aggregates (>1 μm). PE particles spiked to fish were also investigated. Organic residues present during the elution led to an elevated light-scattering signal, complicating the detection [144]. The described procedure was successfully applied to study well-dispersed, spherical polystyrene NPs, but the authors emphasized that these were not a perfect model system for nanoplastics present in the environment. The fact that the method was not successful for other types of nanoparticles (PE) suggested that different methods may be needed for different types of nanoplastics. For the detection and identification of polymeric nanoparticles, Raman spectroscopy, pyrolysis-GC-MS or other MS techniques could be used as alternative methods [161,163].

Although, a multi-detection AF4 platform is very useful to identify structural and morphological differences resulting from aggregation/agglomeration or from interactions of the NPs with contaminants present in complex media, careful optimization of the AF4 method is necessary. Particle-particle interactions due to the carrier liquid (e.g. carrier salinity) and interactions with the ultrafiltration membrane can cause variations in the elution time, which impairs accurate analysis and may lead to low recoveries [72,103,164–166]. Various studies have described these problems and focused on the optimization of the carrier-liquid composition (ionic strength, pH, type of salt; added surfactant). The authors inferred that method development and optimization is vital for each specific type of NPs [138,166–170].

5.2. Interactions involving nanotherapeutics

The use of nanotherapeutics as drug-delivery systems has sparked an ever-growing interest in the field of medicine [171]. Some of the investigated types of drug-delivery nanocarriers include polymeric nanoparticles, micelles, dendrimers, polymersomes, liposomes, virus-like particles and metallic nanoparticles [172]. The characterization of many of these carriers requires mild separation and near-native conditions, due to the fragility of their structures. The composition, size, shape and physicochemical properties of the nanocarriers play a significant role in their stability in biological media, their ability to load and release drugs, and in the kinetics of the drug release. A wide variety of analytical tools are necessary for the detailed characterization of the nanocarriers.

Preclinical physicochemical characterization of nanotherapeutics is crucial to establish their biodistribution, pharmacokinetics, and safety profiles. The behaviour of nanotherapeutics in biological media (e.g. serum, plasma) is often difficult to predict, due to transformations and the interactions between the various components present in such media, which may lead to the formation of a protein corona. Encapsulation of drug molecules in the nanocarrier may improve not only the immunogenicity, biocompatibility, and biodegradability of a compound, but also influence localization in the target cell. Close monitoring of the encapsulation process and possible structural and morphological changes in the nanocarrier particles are important. AF4 has been reported in a number of articles as a suitable technique for the fractionation and characterization of nanocarriers [16,50–52,173]. In the last few years, the value of FFF for the field of nanotechnology started to attract the attention and recognition of the regulatory agencies. The first ISO standard method for the analysis of nano-objects using AF4 and CF3 (ISO/TS 21362:2018) was approved in 2018. A standardized protocol for the determination of the size distribution of medical nanoparticles, such as liposomal drug formulations, using AF4 with on-line size and concentration detectors, was developed by the USA Nanotechnology Characterization Lab (NCL-NCL) and the European Nanomedicine Characterisation lab (EUNCL), and was published by ASTM International (ASTM WK68060) in 2019 [174]. One of the earliest mentions of field-flow fractionation techniques for not only measuring size and size distribution, but also interactions between a drug and its delivery system was presented by Fraunhofer et al. [175]. In this work the behaviour of gelatin nanoparticles as possible drug-delivery carriers was investigated. AF4 and SEC coupled to MALS were used to fractionate the gelatine bulk material, to provide detailed characterization of the gelatine nanoparticles and to monitor the drug-loading process of the nanoparticles. The estimated fraction of HMW components present in different batches was similar between the two techniques. However, it appeared that in SEC the HMW gelatine specimen was degrading due to the shear forces exerted by flow gradients in the narrow channels between the stationary phase particles. Based on these results, AF4 was considered a more suitable technique for monitoring the drug-loading process. The gelatine particles were further loaded with an oligonucleotide (5.4 kDa) and subjected to AF4 analysis. Comparison between the fractograms of the unloaded and loaded nanoparticles showed an increase in the UV absorbance
of the oligonucleotide-loaded particles. Since then, AF4 has been used not only as a sizing technique, but also as a tool to study the stability of nanocarriers and their aggregation/agglomeration behaviour under various conditions, the drug-encapsulation process and resulting structural changes, and changes in the surface modifications and protein-corona formation in the presence of biological media. Determination of nanoparticle-loading efficiency by conventional methods is quite laborious and AF4 may provide a faster, yet accurate alternative. The use of AF4 to characterize the behaviour of different types of drug-delivery nanocarriers (polymeric nanoparticles, liposomes, virus-like, and metallic NPs) will be discussed next. A summary of the discussed drug delivery systems, with the AF4 conditions and the detectors used to study the drug loading and release, the drug transfer kinetics and the resulted structural and morphological changes can be found in Table 4.

5.2.1. Polymeric nanocarriers for drug delivery
Polymeric nanoparticles are mainly composed of biodegradable and biocompatible polymers of synthetic or natural origin. Poly(-lactic acid) (PLA), poly(lactide-co-glycolide) (PLGA), and polycaprolactone (PCL) are some synthetic polymers frequently used as nanocarriers. Among the natural polymers used are alginate, albumin, and chitosan [176]. Polymeric nanocarriers are the protective shell for encapsulated therapeutics (drugs, genetic material, etc.), facilitating their cellular penetration and endo-lysosomal release.

AF4 allows accurate determination of the loading efficiency of the polymeric nanocarriers, as discussed in the reviews of Wagner et al. and Quattrini et al. [50,52]. Several of the techniques used for separating and isolating the drug from the carrier, such as ultrafiltration and centrifugation, may degrade the nanocarriers and promote drug release [177,178]. AF4 provides “mild” separation conditions [179] and, when coupled to suitable detectors (UV absorbance, dRI, FLD, MALDI, ICP-MS, etc.), allows direct (measuring the drug in the carrier) or indirect quantitation (from the unbound drug in the supernatant) of the encapsulated drug [180].

Different quantitation approaches may be followed, depending on the size and properties of the investigated drug (e.g. macromolecule, small-molecule drug, genetic material). If the drug is a macromolecule separation between the unloaded (free) drug and the encapsulated nanoparticle can be achieved by AF4. The response of concentration detectors (UV/dRI) allows estimation of the concentration of the unloaded drug molecules. Drug molecules of low or intermediate molecular weight that are weakly bound may pass through the ultrafiltration membrane. Two possible solutions have been considered for the detection of such small molecules, viz. i) addition of detergent in the carrier liquid to create larger drug-complexes and maintain these during fractionation [181] and ii) collection of the filtrated through the membrane eluent for in-direct quantification of the unloaded drug [182].

The encapsulation and drug-release processes of stimuli-responsive polymersomes have been studied with AF4 under various conditions (e.g. temperature, pH, redox conditions) [183,184]. It is also imperative to know where the cargo macromolecule has been attached (e.g. inside the membrane bilayer or in the core). There are two ways of cargo encapsulation for stimuli-responsive polymersomes. The drug molecule can be loaded either during self-assembly (preloading) or afterwards (postloading). Gumz et al. [36] used AF4 coupled to DLS and MALS to study the location of the encapsulated protein (myoglobin and esterase). DLS showed that the size of the polymersomes was not influenced by the protein incorporation. However, changes in the membrane roughness were observed. The slope of the scaling plot corresponds to the exponent (x) in the scaling law \( R_g \sim K M^x \), where \( R_g \) is the radius of gyration, \( M \) is molecular weight and \( K \) is a constant. Such plots provide information on the shape and conformation of particles. Changes in the membrane roughness were indicated by an increase in \( n \). The effect was more apparent for esterase than for myoglobin, suggesting that myoglobin was able to penetrate the core, whereas esterase remained in the outer layer.

Dye molecules are small molecules often employed as model compounds to study and monitor the encapsulation process in polymeric nanocarriers. AF4 coupled to MALS and fluorescence detection (FLD) appears to be particularly useful to monitor not only the drug encapsulation, but also possible interactions between the released drug and plasma proteins [185,186]. In real conditions, nanocarriers have to be transferred through the blood stream to the target organ. Premature drug release and binding between the drug and plasma proteins may occur. In a recent study, Trindade et al. [185] used AF4-MALS-FLD to evaluate four different polymeric “nanocapsules” (PCL, PLA, PLGA, PLA-PEG) for their drug-binding properties in serum. Only minor transfer of the fluorescent dye to serum proteins was detected, even during long incubation times (2–12 h), showing that the dye molecule was largely retained within the nanocarrier even in the presence of serum proteins.

The morphology of nanoparticles is known to play a significant role in binding to their target, but also in the efficiency of the drug delivery. AF4 allows fractionation of nanocarriers based on their morphological features, which may help understand the conditions necessary for controlled fabrication of nanocarriers with well-defined architectures [35,187]. The shape factor \( \rho = R_g/R_b \) and the fractal dimension (\( \delta \)) can be estimated as morphological indicators from the AF4-MALS and DLS data. For hard spheres \( \rho \) is close to 0.778, while particles with hollow spheres or with high aspect ratios show values above 1. The fractal dimension is obtained from a logarithmic plot of molecular weight vs. \( R_g (\bar{M} = \bar{R}_g^3) \) and is related to the compactness of the carrier. For densely packed particles or aggregates high \( \delta \) values (>2.5) are expected, whereas branched structures and rod-like particles show lower values (\( \delta \leq 1 \)) [35]. Gennari et al. [158] used AF4 to investigate the morphology of hyaluronic acid (HA) and chitosan nanoparticles formulated in two different ways, viz. (i) Formation of the nanoparticles upon mixing of the two polymers (direct complexation) or (ii) by addition of a triphosphate (TPP) template to promote gelification of chitosan before adding HA (templated). Although the fractal dimension was in all cases above 2, indicating the formation of compact (spherical) particles, some differences were observed. The template chitosan/HA particles showed lower \( \delta \) values (Fig. 3) indicating that the template process resulted in a less-dense HA shell around the chitosan core.

5.2.2. Liposomal nanocarriers for drug delivery
Liposomes are composed of lipids and show good biocompatibility, biodegradability, minimal cytotoxicity. They also allow encapsulation of various cargo molecules [188]. Their physicochemical properties can easily be adjusted by varying the lipid composition during self-assembly. Conventional liposomes consist of a lipid bilayer with an aqueous centre, allowing encapsulation of both hydrophilic and hydrophobic drugs. Further optimization can be achieved through surface modifications, by binding PEG, antibodies, peptides, proteins, or combinations thereof [189].

AF4 with a multi-detector system has long been established as a tool for accurate determination of the size, shape, surface charge and stability of liposomes [190–194]. Moreover, AF4 has been used to study the drug-encapsulation efficiency and drug-release kinetics of liposomes.

Kuntsche et al. [195] studied the optimal selection of carrier vesicles based on drug characteristics. Quantitative information about the sample recovery was obtained by fractionation of radio-labelled (\(^3\)H or \(^14\)C) drug-loaded liposomes. An in-depth
Examples of studies involving interactions between nanoparticles and drug-related compounds using AF4.

<table>
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<th>Polymeric Nanocarriers</th>
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<th>Additional techniques used in the study</th>
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<th>Ref.</th>
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<tr>
<td>Polymersomes + Myoglobin/ Esterase</td>
<td>AF4-UV/Vis - MALS-drF-LD 490 µm spacer RC membrane (10 kDa MWCO) F&lt;sub&gt;e&lt;/sub&gt;: alternating F&lt;sub&gt;s&lt;/sub&gt;: 1.0 mL min&lt;sup&gt;-1&lt;/sup&gt; F&lt;sub&gt;d&lt;/sub&gt;: 0.5 mL min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>100 mM PBS</td>
<td>cryo-TEM, enzymatic assays</td>
<td>Loading efficiency was found to depend on the loading approach and the type of loaded protein</td>
<td>[36]</td>
</tr>
<tr>
<td>Polymersomes + Polymersomes</td>
<td>AF4-UV/Vis-MALS-FLD 350 µm spacer RC membrane (5 kDa MWCO) F&lt;sub&gt;e&lt;/sub&gt;: 1.5, 2.0 mL min&lt;sup&gt;-1&lt;/sup&gt; F&lt;sub&gt;s&lt;/sub&gt;: 0.5 mL min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10 mM NaCl</td>
<td>AFM, DLS, NTA</td>
<td>AF4 to study the kinetics of dye transfer to serum proteins</td>
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<tr>
<td>Hyperbranched poly(ethylene imine) with maltose shell (PEI-Mal) + dye</td>
<td>AF4-UV/Vis-MALS-drF</td>
<td>n/a</td>
<td>SEC</td>
<td>Interactions between PEI-Mal polymer and packing of SEC column</td>
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<td>Liposomal nanocarriers</td>
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<td>Liposome + p-THPP (lipophilic model drug)</td>
<td>AF4-UV/Vis-MALS-drF 350 µm spacer n/a membrane F&lt;sub&gt;e&lt;/sub&gt;: 2.0 mL min&lt;sup&gt;-1&lt;/sup&gt; F&lt;sub&gt;s&lt;/sub&gt;: 1.0 mL min&lt;sup&gt;-1&lt;/sup&gt; F&lt;sub&gt;d&lt;/sub&gt;: 0.5 mL min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10 mM Tris, 0.02% (w/v) NaN&lt;sub&gt;3&lt;/sub&gt; (pH 7.4)</td>
<td>cryoTEM, DLS, UV/Vis spectroscopy, HPLC-UV/Vis, NMR</td>
<td>AF4 used to determine drug transfer from a (small) liposomal carrier to a large acceptor liposome (model of biological membranes)</td>
<td>[198, 230]</td>
</tr>
<tr>
<td>Liposome + antimicrobial peptides</td>
<td>AF4-UV/Vis-MALS-drF 350 µm spacer</td>
<td>5 mM NaCl, 0.001% Tween20</td>
<td>DLS</td>
<td>Quantification of p-THPP performed both on-line with UV/Vis and off-line with HPLC</td>
<td>[231]</td>
</tr>
<tr>
<td>Liposome + temoporfin (mTHPC) (strong lipophilic drug)</td>
<td>AF4-UV/Vis-MALS-drF 500 µm spacer</td>
<td>150 mM NaCl, 10 mM Tris, 0.03% (w/v) NaN&lt;sub&gt;3&lt;/sub&gt; (pH 7.4)</td>
<td>DLS, LSC, PCS</td>
<td>Lipid radiolabel added to the loaded liposomes to investigate the mechanism of transfer</td>
<td>[202, 232]</td>
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<tr>
<td>Viruses and virus-like particles (VLPs)</td>
<td>AF4-UV/Vis-MALS-drF 350 µm spacer</td>
<td>10 mM Tris–HCl, 50 mM NaCl, 10 mM CaCl&lt;sub&gt;2&lt;/sub&gt; (pH 7.5)</td>
<td>SDS-PAGE, Refractometry, TEM</td>
<td>AF4 showed that encapsulation of VLPs with DNA or non-viral protein may decrease the radius of gyration of the particles</td>
<td>[212]</td>
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**Table 4**

Examples of studies involving interactions between nanoparticles and drug-related compounds using AF4.
investigation of the impact of the fractionation conditions (flow conditions, sample mass, carrier liquid) on the liposome recovery and potential loss of the drug during separation demonstrated that AF4 could be used to monitor drug release and to provide accurate pharmacokinetic information. Results suggested that neither the sample concentration nor the cross-pharmacokinetic information. Results suggested that neither the AF4 could be used to monitor drug release and to provide accurate and potential loss of the drug during separation demonstrated that conditions, sample mass, carrier liquid) on the liposome recovery whereas the more-hydrophobic temopor corticosterone was completely released (recovery about 2%), whereas the more-hydrophobic temopor was well retained (recovery about 80%). Therefore, AF4 seems to be most useful to perform drug-release and drug-transfer studies of lipophilic drugs.

Hinna et al. [196–198] demonstrated the use of AF4 to measure the release and transfer of a hydrophobic model compound (photosensitizer meso-tetra(hydroxyphenyl porphyrin; p-THPP) from small “donor” liposomes (70–80 nm) to co-incubated large, empty “acceptor” liposomes (approximately 450 nm), which served as a model of a biological “sink” (e.g. plasma proteins, lipoproteins, cell membranes). The model-drug contents in the donor and acceptor AF4 fractions were quantified by on-line measurements of UV/Vis extinction and by off-line HPLC analysis of collected fractions. The release and transfer kinetics of the drug (p-THPP) were determined after sampling at specific incubation times. It was suggested that the transfer of p-THPP was following first-order kinetics with a half time of about 300 min. This methodology was also used to study the kinetics of drug release of several lipophilic drugs and the

Table 4 (continued)

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<tr>
<th>Analytes</th>
<th>FFF Conditions</th>
<th>Eluent</th>
<th>Additional techniques used in the study</th>
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<tr>
<td>Human polyoma JC VLPs + DNA</td>
<td>AF4-UV/Vis-MALS-DLS-dRI</td>
<td>10 mM Tris–HCl, 50 mM NaCl</td>
<td>Encapsulation of the non-viral protein prohibited the co-encapsulation of DNA</td>
<td>AF4 used to monitor the VLP dissociation and DNA encapsulation</td>
<td>[204]</td>
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<tr>
<td>Enveloped bacteriophage φ6</td>
<td>AF4-UV/Vis-MALS</td>
<td>10 mM PB, 150 mM NaCl, SDS-PAGE, SEC, EM, IEC,</td>
<td>AF4 to study the controlled dissociation of φ6 virus under various conditions</td>
<td>Active viral subassemblies were collected after AF4 fractionation</td>
<td>[210]</td>
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<tr>
<td>Q beta bacteriophage VLPs</td>
<td>AF4-UV/Vis-MALS</td>
<td>Deionized water</td>
<td>CyEffFF used to separate unmodified VPLS and VLP-peptide conjugates</td>
<td>CyEffFF identified additional sub-populations of aggregated VLPs not identified by AF4</td>
<td>[215]</td>
</tr>
<tr>
<td>Metallic nanoparticles</td>
<td>Gold nanoparticles (AuNPs) + antibodies</td>
<td>AF4-UV/Vis-MALS-dRI</td>
<td>PBS, 0.005% Polysorbate 20 (Tween-20)</td>
<td>Stability and aggregation state of concentrated conjugates of AuNPs with antibodies were monitored by AF4</td>
<td>[233]</td>
</tr>
<tr>
<td></td>
<td>Deionized water</td>
<td>DLS, ELS, ICP-MS, TEM</td>
<td>Non-specific binding between conjugates in mixed samples was not observed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metal-organic frameworks (nanoMOFs) + nucleoside reverse transcriptase inhibitor (NRTI)</td>
<td>AF4-UV/Vis-MALS-dRI</td>
<td>4 mM NH4HCO3, 0.02% Tween20 (pH 10)</td>
<td>AF4 used to monitor nanoMOFs morphological changes as indicators of their interaction with the drug molecules</td>
<td>Encapsulation of the drug molecules may increase the root-mean-square radius of the nanoMOFs</td>
<td>[234]</td>
</tr>
<tr>
<td></td>
<td>Deionized water</td>
<td></td>
<td></td>
<td>Number of phosphate groups present in the drug molecules affected the binding with the nanoMOFs, indicating the nature of the binding mechanism</td>
<td></td>
</tr>
<tr>
<td>CdSe/ZnS core-shell quantum dots (QDs) + antibody</td>
<td>AF4-ICP-MS</td>
<td>4 mM NH4HCO3, 0.02% Tween20 (pH 10)</td>
<td>AF4-ICP-MS to identify the bioconjugation reaction products and to quantify nanoparticle:antibodies bioconjugation efficiency</td>
<td></td>
<td>[225,227]</td>
</tr>
</tbody>
</table>
Influence of varying the donor-acceptor ratio [197].

Conventional methods for determining drug content require breakdown of the liposomal structure [199]. Consequently, crucial information regarding the location of the drugs or excipients or regarding the influence of the liposome size cannot be obtained. With an increase of liposomal size, membrane curvature is decreased and differences in the lipid packing between outer and inner membrane also decrease, which may influence the drug partitioning. AF4 has been used to determine distributions of drugs (temoporfin and amphotericin B) and excipients (cholesterol) in liposomes of different sizes [199–201]. In the work of van Haute et al. [199] liposomes were fractionated with AF4 and the particle size of each fraction was determined with nanoparticle-tracking analysis (NTA). Off-line HPLC with UV/Vis and charged-aerosol detection (HPLC-UV/Vis-CAD) was used for quantitation of the drug (amphotericin B) and the lipid excipients and for estimation of the drug-to-total-lipid ratios of the various fractions. A four-fold increase in this latter ratio was observed with increasing liposome size from 75 to 125 nm.

The interaction of liposomes with plasma proteins is very complex and can significantly affect the stability of the nanoparticles and alter their properties. Drug release form the liposome. Drug recovery of doxorubicin was nearly 65% of incubation with plasma. The dual-loaded liposomes were fractionated with AF4 and the particle size, size distribution and presence of fragments, oligomers, and aggregates of viruses and VLPs, because the native structure and viral infectivity of the analysed particles can be preserved [56]. AF4 has been used to study the particle size, size distribution and presence of fragments, oligomers, and aggregates of viruses and VLPs. AF4 has also facilitated studies related to the stability of VLPs [205–208], the virus assembly/disassembly process [204,209–211], and the encapsulation of heterologous DNA [204,212]. In these experiments, the AF4 separation was followed by MALD (information about mass and Rv) or DLS (information about Rn) detection, and concentration detectors such as UV/Vis absorbance (single wavelength or diode-array detector, DAD) or DRI for quantification of the various species.

5.2.3. Viruses and virus-like particles for drug delivery

Viruses and virus-like particles (VLPs) are increasingly used as gene-therapy delivery vehicles, due to their versatility and safety. Such bioparticles intended for vaccination or gene therapy need to be pure, stable, homogeneous in size and free from aggregated species. Viruses, VLPs and their subassemblies are large macromolecular complexes, and their purification and characterization pose great challenges. In addition to the virus or VLPs particles, often other proteins, DNA, lipids, and cell debris, ranging in size from nm to μm are present in the sample. SEC is not well suited for samples with such a high level of size heterogeneity, due to the limited size range covered [204]. AF4 is an attractive alternative for purification, identification, and characterization of viruses and VLPs, because the native structure and viral infectivity of the analysed particles can be preserved [56]. AF4 has been used to study the particle size, size distribution and presence of fragments, oligomers, and aggregates of viruses and VLPs. AF4 has also facilitated studies related to the stability of VLPs [205–208], the virus assembly/disassembly process [204,209–211], and the encapsulation of heterologous DNA [204,212]. In these experiments, the AF4 separation was followed by MALD (information about molar mass and Rv) or DLS (information about Rn) detection, and concentration detectors such as UV/Vis absorbance (single wavelength or diode-array detector, DAD) or DRI for quantification of the various species. To monitor the encapsulation of a foreign protein into murine-polyomavirus VLPs, AF4-MALS, TEM, and electrospray—differential-mobility analysis (ES-DMA) were used [213]. To explore the capabilities of the three techniques to detect subtle size differences, a) an empty VLP, b) a VLP packaged with a foreign protein, c) a VLP incorporating genomic DNA, and d) a VLP with both
genomic DNA and a modified surface were compared. ES-DMA and AF4 were both able to differentiate between (a) and (b), between (a) and (c), and between (c) and (d) with greater statistical significance than TEM. Shifts in size and size distributions between the various VLPs were monitored with AF4-MALS, and insights were obtained in the internal packaging of nucleic acids and the chimeric incorporations of surface proteins. ES-DMA and AF4 consistently showed slightly deviating results for all VLP modifications, most likely due to differences between the in-solution and dry states of the virus particles. This indicates the need for complementary techniques to ensure quality in the development and production of innovative vaccines and gene VLP carriers. Reports using TEM also showed that various VLPs could not be morphologically distinguished [214]. However, later studies using AF4-MALS showed that VLPs undergo not only size changes, but also morphological changes upon packing with DNA and proteins [204,212,213].

Recently, another FFF sub-technique, cyclical-electrical-field flow fractionation (CyEIFFF) was reported for simultaneous characterization of size and surface charge of Q-beta-bacteriophage VLPs that differed in the surface conjugated peptide [215]. CyEIFFF fractionated the VLPs based on their surface electric charge (electrophoretic mobility), providing additional resolution between the subpopulations in comparison with AF4. However, the poor resolution between the VLPs and their aggregates proved a considerable limitation of the technique.

5.2.4. Metallic nanoparticles for drug delivery

The use of (noble-)metal-based nanoparticles has exploded for medical applications, such as bioimaging, biosensors, targeted drug delivery, photoablation and radiation therapy [216]. Modification and functionalization of these particles with specific functional groups allows them to bind to antibodies, drugs or other ligands, making them highly promising for biomedical applications and cancer therapy. Functionalization of the surface of the nanoparticle with polymers or proteins creates conjugated systems to improve biocompatibility, stability, and control of toxicity. AF4 coupled to suitable detectors has been used for characterizing size and morphology [217], and for studying the aggregation/agglomeration [218] and transformation [219–222] of metal nanoparticles and their conjugates in complex media.

The aggregation behaviour of cytochrome-C-conjugated silver nanoparticles (cyto-C-AgNPs) under various conditions (pH, temperature, ionic strength) was investigated by AF4 [223]. The effects of storage time, temperature and ionic strength on the recovery of cyto C-AgNPs were measured to study in-solution aggregation of the particles. It was concluded that partially unfolded cytochrome-C adsorbed on the surface of the AgNPs led to interaction with the protein molecules on other AgNPs, resulting in aggregation.

The capabilities of AF4 in probing interactions between multifunctional gold nanoparticles (AuNP) and biomolecules were described by Ojea-Jiménez et al. [222]. Multifunctional nanoparticles are especially attractive for diagnostic and therapeutic applications, thanks to the multiple binding chemistries of their surface. The effect of the coating density of a ligand (hydroxy-terminated poly(ethylene oxide); PEO-OH) on non-specific protein adsorption was investigated. Online coupling of AF4 to DLS provided valuable insights in changes in hydrodynamic diameter and amounts of PEO attached to the surface of AuNPs, when varying the stoichiometry of the reaction mixture. Combination of the hydrodynamic diameter ($R_h$) obtained by AF4-DLS and data from centrifugal liquid sedimentation (CLS) yielded densities of the AuNP-ligand complexes [217,222]. To study the effect of coating density on protein adsorption, AuNP and AuNP-PEO-OH particles were incubated with human serum albumin. The functionalized AuNPs showed no increase in size as a result of the protein binding. Results showed that the PEO-coating reduced the protein adsorption, while the number of covalently attached proteins could be controlled by the number of available carboxyl groups on a bi-functional AuNP with PEO-COOH/PEO-OH ligands [222].

The influence of the surface chemistry of quantum dots (QDs) on their agglomeration in biological media was also investigated with AF4-MALS and DLS [218]. The agglomeration of QDs of identical size, but coated with three different negatively charged ligands (mercaptoethanol, ATRPMA; dihydroxyacid, DHLA; carboxylated-poly(ethylene glycol), PEG-COOH), was studied. The particles were incubated for various times in water, and cell media with and without serum. The different ligands caused distinct differences in agglomeration behaviour of the QDs. The QD-PEG-COOH particles showed no sign of agglomeration in any of the examined media. It was concluded that the PEGylated ligand can improve the stability of a formulation due to steric effects between the hydrated PEG-chains, and electrostatic repulsion between the negatively charged end groups. The chemistry of the ligands also determines their ligand-packing density. Ligands with a high packing density (such as MPA) enhanced the stability of the QDs in serum compared to ligands with a low packing density (such as DHLA). The low packing density of DHLA allowed protein adsorption, favoring the formation of a protein corona. AF4 provided accurate size information of QD agglomerates in biological matrices, enabling quantitative determination of the extent of agglomeration [218]. Coupling of AF4 with the element-specific ICP-MS was used to study the covalent conjugation between antibodies and QDs [34,224–227]. AF4-ICP-MS was used for determining the ratios between CdSe/ZnS core–shell QDs and the rat monoclonal IgG2a antibodies (mAb) attached to their surface after chemical functionalization [225]. Upon fractionation of the bio-conjugate particles with AF4, ICP-MS was used to obtain metal-to-sulfur ratios along the peak, enabling the calculation of the average QD-to-antibody ratio and revealing the presence of various bio-conjugated QDs. The AF4-ICP-MS platform can be used to investigate the conjugation between various metal-containing nanoparticles with different (bio)molecules and establish the optimal functionalization [34].

5.3. Protein corona formation

Nanocarriers that are used for drug delivery come in contact with biological fluids and adsorption of proteins on their surface may lead to the formation of nanoparticle-protein complexes or “protein corona”. This may influence the stability of the nanoparticles in the biological environment and ultimately affect their opsonization, cell localization, and trafficking qualities [235]. Therefore, understanding and monitoring of the association between nanoparticles and proteins is important to predict their fate when introduced in the human body and to develop safe and reliable nanomedicines.

Depending on the binding affinity of the proteins adsorbed on the surface of the nanoparticle, two types of coronas are distinguished. High-affinity proteins adsorbed on the nanoparticle surface form a “hard corona”, whereas low-affinity proteins form a “soft corona” [235]. The nanoparticle-protein complex is a dynamic system and separation of the particles from the biological fluid, while preserving the integrity of the corona formed, is challenging. Centrifugation, followed by several washing steps, is often used to separate the corona from the free plasma proteins. However, there are certain limitations to this approach [236]. For the removal of highly abundant proteins several washing steps are required, otherwise they can be overestimated [237]. However, desorption of the bound proteins or disruption of the nanoparticle structure may occur as a result. Studies have focused on the comparison of
centrifugation and AF4 to determine which of the two techniques can best preserve the “hard” and “soft” protein coronas [237, 238]. Ashby et al. [238, 239] studied this issue for superparamagnetic iron-oxide nanoparticles (SPIONs) using analytical centrifugation and AF4, followed by LC-MS/MS for identification. SPIONs were first incubated with IgG/albumin-depleted serum to form the protein corona. It was assumed that dissociation of the soft corona would occur in AF4. Centrifugation was performed with only one washing step to precipitate all proteins of the entire corona. It was concluded that both AF4 and centrifugation could isolate proteins with good binding affinity to the nanoparticles (hard corona). However, AF4 was thought to remove proteins from soft coronas, with relatively fast association rates, preventing their re-association to the particles. Weber et al. [237, 240] used AF4 and analytical centrifugation to monitor the protein corona formed by PEGylated polystyrene nanoparticles incubated in human blood plasma (a medium containing free proteins) as shown in Fig. 4. The nanoparticle-protein complex was isolated after centrifugation and after AF4. The protein composition was determined by SDS-PAGE and LC-MS and it was found to differ greatly between the two separation methods (Fig. 4; right). The data obtained after centrifugation showed higher concentrations of lipoproteins. In contrast, the most abundant protein found after AF4 separation was human serum albumin (HSA), which is known to form a soft corona. Further analysis of the centrifuged samples with AF4 revealed that the hard protein corona can be preserved by both techniques. However, conclusive information on whether or when AF4 may be used to study soft coronas does not yet seem to be available.

AF4 can assist in the characterization of the nanoparticle-protein corona complexes in two ways.

1) To study the protein-corona formation by comparing the differences in size and size distribution before and after incubation in the plasma or serum.

2) To separate the nanoparticle-protein complex from the free-plasma proteins, prior to a proteomic-analysis approach (LC-MS/MS, electrophoresis).

However, one of the main challenges in AF4 is to achieve sufficient separation between the mixture of free proteins and the drug-delivery nanocarriers. The different chemical nature of these analytes requires specific carrier liquids in AF4 in order to prevent analyte-membrane interactions and ensure good recoveries and optimal separation [241]. To avoid nanoparticle-membrane interactions, a surfactant-based carrier liquid is often necessary. However, this may cause overestimation of the particle sizes and micelle formation, complication the detection and understanding of the nanoparticle-protein-complex formation [242]. An increase in apparent size of the nanocarriers may result from protein-corona formation or from aggregation/agglomeration of the particles [220]. In a recent study [243] a thorough protocol was described that included control runs of both the plasma and the nanoparticles separately (negative controls) under the same conditions as the plasma-incubated polymeric nanoparticles. Identification of the proteins in the fractions was based on label-free quantitative proteomics (SDS-PAGE followed by LC-MS). Comparison between the negative controls and the plasma-incubated particles allowed accurate assignment of the bound-proteins, excluding coelution events or presence of contaminants. Three cross-linked polymeric nanoparticles with hydrophilic coatings were incubated in human blood plasma and analysed for protein-corona formation [243]. The number of proteins found to be associated with the nanocarriers was essentially negligible. Although there is a considerable chance that proteins are only weakly bound to the nanocarriers and dissociate during analysis, the long circulation times of these synthetic nanocarriers in plasma suggests that they may be effectively resistant to protein-corona formation. In Table 5 examples of studies involving the use of AF4 to monitor the protein corona formation are summarized and the main conclusions are mentioned.

Recently, CF3 coupled to DLS was demonstrated as a tool to study the adsorption of specific antibodies against immunoglobulins of type E (algE) on polystyrene NPs [244]. Information obtained by CF3-DLS and centrifugal particle separation (CPS) allowed determination of the density of the adsorbed antibodies on the surface of the NPs. Incubation of the functionalized algE-polymer NPs with immunoglobulins showed a mass increase. However, the aggregation of particles and molecules disturbed the CPS signal impairing conclusions on the stoichiometry. Further investigation and optimization of the experimental procedures was considered necessary.

Fig. 4. Left: Overlaid fractograms of human plasma (blue trace), polystyrene NPs (green trace), polystyrene NPs incubated in plasma (red trace). Right: Comparison of the composition of the protein coronas preserved after centrifugation or after AF4, analysed by LC-MS. The identified proteins were grouped according to their function. Figure reprinted (adapted) from *Acta biomaterialia*, Vol. 76, Weber, C., Simon, J., Mailander, V., Morsbach, S. and Landfester, K., Preservation of the soft protein corona in distinct flow allows identification of weakly bound proteins, pp.217-224 (ref. [237]). Copyright © 2018, with permission from Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
6. Concluding remarks and recommendations

AF4 is the most widely used among the FFF techniques, with valuable applications for the characterization of a wide variety of analytes, such as (bio-)macromolecules and micro- and nanoparticles. AF4 is an established sizing technique. The aim of this review was to provide a detailed overview of studies in which information obtained by FFF techniques (e.g., size, density, molar mass, shape, metal composition), coupled to a wide variety of detectors, were used to study and monitor intermolecular interactions in very diverse matrices. Figs. 5 and 6 provide an overview of most of the subjects that have received most attention in the literature during the last 15 years. Fig. 5 shows the main technical aspects of AF4 that are discussed in various sections. It is clear from this figure that the attractiveness of FFF as a separation technique arises largely from its hyphenation with a great variety of detectors. Fig. 6 summarizes the kinds of samples that we found to have been subjected to AF4 and the types of interaction studies performed. Other types of FFF, such as TF3 and CF3/SdFFF, are more sporadically used for studying interactions. TF3 was shown to be useful to study the association of polymers (aggregation or self-assembly) mostly in organic solvents, with only few applications for aqueous solutions. CF3/SdFFF can be used to study the kinetics of the aggregation of emulsions, and it appeared to be promising for monitoring the formation of protein corona. Hyphenation of AF4 with a variety of detectors enables monitoring of structural, morphological and chemical alterations that result from inter- and intramolecular interactions. Changes in size, shape, density, aggregation state, as well as changes in elemental composition of the particles over time under various conditions (pH, ionic strength, temperature etc.) can be observed. The absence of a packing in AF4 channels offers important advantages for molecular interaction studies. An overview of some key advantages, but also some difficulties that have to be considered is provided in Table 6. Polydisperse samples can be analysed, which is particularly useful for the separation of proteins or polymers from their aggregates/agglomerates [66] or self-assemblies [21], or for the separation of bioactive compounds from drug delivery carriers [237]. Shear-sensitive structures that are prone to dissociation may be preserved during the separation in the open-channel allowing an accurate understanding of their in-solution state [31,98,237]. A wide range of solvents or buffers can be used to investigate the stability.
**Fig. 5.** Main technological aspects of AF4 discussed in the review.

- **Concentration detectors**
  - UV/Vis absorbance allows monitoring drug loading and release (for drugs with strong chromophores)
  - Diode-array detection (DAD) allows simultaneous detection of multiple targets
  - Refractive-index detection (dRI) is mainly used for non-UV-active polymers
  - In combination with MALS dRI yields information on the molar mass
  - Both UV/Vis and dRI allow quantification of analytes
  - Fluorescence detection (FLD) allows monitoring of binding affinity and dissociation constant (Kd) using fluorescently labelled molecules
  - FLD allows monitoring of drug loading and release

- **MALS**
  - Allows accurate determination of molar mass / molecular weight (MMW), radius of gyration (Rg) and fractal dimension (δ)
  - Allows estimation of the stoichiometry of protein complexes and information on the aggregation kinetics

- **DLS**
  - Determination of hydrodynamic radius (Rh)
  - In combination with MALS data estimation of the shape factor (Rh/Rg)

- **ICP-MS & single-particle ICP-MS**
  - Monitoring changes in the elemental composition to study interactions involving inorganic NPs or metalloproteins
  - Targeting sulfur can be used for quantification of antibodies in biocomplex particles
  - Detection limit in the ng L\(^{-1}\) range
  - Accurate information on molar mass of molecules and complexes
  - Potentially provides information on lipophilic and amphiphilic compounds within liposomal carriers

**Fig. 6.** Schematic overview of a) the types of matrices and b) the types of interactions that have been studied with AF4 and are discussed in this review.

- **Biological samples**
  - Separation of whole blood (including blood cells), plasma and serum with minimal sample pre-treatment
  - Analysis of bioactive compounds in the targeted environment

- **Cosmetic products**
  - Separation and characterization of food-related macromolecules
  - Quantification of NPs
  - Structural and chemical modifications of NPs in realistic matrices
  - Stability of NPs

- **Food and drink products**
  - Impact of structural and chemical modifications on toxicity of NPs
  - Interactions with other components present (e.g., NOM)
  - Stability of NPs

- **Soil and water samples**
  - Quantification of inorganic NPs
  - Structural and chemical modifications of NPs in soil and water matrices
  - Stability of NPs

- **Aggregation**
  - Various stressors (pH, ionic strength, etc.)
  - Structural and conformational changes
  - Separation of nano- and micro-meter particles
  - Stoichiometry of complexes
  - Information on aggregation kinetics

- **Polymer association**
  - Association of copolymers of different compositions and architectures
  - Separation of structures of different morphologies
  - Characterization of ultrahigh-molecular weight polymeric structures

- **Polymer-protein conjugation**
  - Affinity between polymer and protein
  - Purification of conjugated systems
  - Stoichiometry of polymer-protein conjugate
  - Investigation of stability

- **Drug encapsulation / release**
  - Quantitation of the encapsulated drug
  - Localization of the drug on the membrane (changes in membrane roughness)
  - Information on release & encapsulation kinetics
  - Interactions between particle or drug and the biological medium

- **Protein corona formation**
  - Investigation of the binding affinity between NPs and blood proteins
  - Distinction between low- and high-affinity proteins (soft and hard protein coronas)
of the molecules/particles.

Investigations of protein-protein interactions and the binding affinity and stoichiometric ratios between different proteins or proteins and other molecules (e.g., polymers, nanoparticles) are possible with AF4. In Table S1 a summary of interaction parameters calculated from information obtained by FFF analysis is presented. Moreover, AF4 was shown to be a useful tool to monitor parameters that must be optimized for each specific application and sample. In certain cases, even after optimization and careful selection of the essential separation parameters (such as flow rate, focusing time, eluent composition), weakly-associated complexes (such as soft protein coronas, labile protein complexes) may be disrupted. Frit-inlet FFF may be a useful alternative for the analysis of labile structures and complexes, by eliminating the focusing process [31,98]. Moreover, particle-particle interactions because of the carrier liquid composition and interactions between analytes and the ultrafiltration membrane have to be taken into account and studied carefully. For certain classes of particles, interactions have been found unavoidable with the currently available membranes [24]. Development of new membrane materials and better characterization of the available ones is important to broaden the application range of AF4.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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J. Bott, R. Franz, Investigation into the potential migration of nanoparticles from laponite-polymer nanocomposites, Nanomater. 8 (2018), https://doi.org/10.3390/nano8090723.


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