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A perspective on the characterization of colloids and macromolecules using asymmetrical flow field-flow fractionation

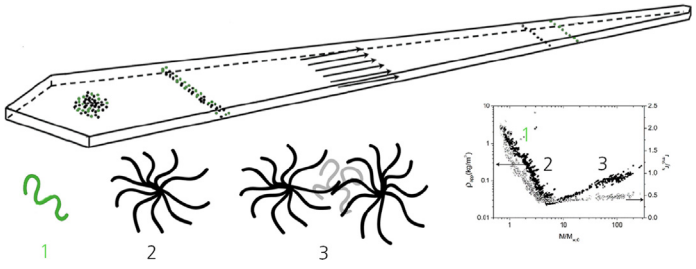
J. Ray Runyon[a,b], Matilda Ulmius[b], Lars Nilsson[a,b,*]

[a] Lund Centre for Field-Flow Fractionation, Department of Food Technology, Engineering and Nutrition, Lund University, Lund, Sweden
[b] SOLVE Research and Consultancy AB, Lund, Sweden

HIGHLIGHTS

- AF4 aids the characterization of the physicochemical properties, such as shape and aggregation state of colloidal materials.
- AF4 is a method of choice for analyzing protein aggregates in therapeutics as recommended by the FDA.
- Aggregated structures of macromolecules are separated and characterized by AF4 providing insight into their functionality.

GRAPHICAL ABSTRACT



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ABSTRACT

Asymmetrical flow field-flow fractionation (AF4) is rapidly becoming a technique of choice for the separation and characterization of complex materials. It is capable of fractionating samples over a wide size (~2 nm to 50 μm in diameter) and molecular weight range (10^3–10^{10} g/mol). It offers gentle, low shear, low pressure separation conditions which are essential to preserve the structure and aggregation of fragile species. In this paper we illustrate a number of examples where AF4 plays central role in providing detailed and accurate characterization of polydisperse and complex colloidal and macromolecular materials such as proteins, polysaccharides, nanoparticles, and emulsion droplets.

1. Introduction

Dispersions of colloidal particles play an important role in food, pharma and material sciences. A fundamental property of colloidal particles is their size as it influences the functional properties (such as colloidal stability and surface area) of the particles. In many cases, populations of colloidal particles are polydisperse and, hence, a range of different particles can be present within the population. Furthermore, the particle populations may contain a mixture of species with differences in both chemical and morphological properties such as shape, conformation and structure. Together these properties put great demand on the characterization work of such colloidal dispersions; obtaining accurate and detailed information of both the size distribution and the functional properties over the size distribution typically requires fractionation prior to characterization.

A powerful fractionation technique that is suitable for this purpose is asymmetrical flow field-flow fractionation (AF4) [1,2]. AF4

* Corresponding author at: Lund Centre for Field-Flow Fractionation, Department of Food Technology, Engineering and Nutrition, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden. Tel.: +46 46 222 83 03.
E-mail address: lars.nilsson@food.lth.se (L. Nilsson).

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is capable of fractionating particles (approximately 2 nm to 50 μm) and macromolecules (10^3–10^{10} g/mol) in a very wide size range giving rise to unique characterization possibilities for colloidal dispersions (when combined with multiple detection schemes). AF4 has many similarities to chromatography but one major difference is that instead of a packed chromatography column AF4 utilizes a separation channel containing no packing material. The open channel gives rise to relatively low pressures, low shear and gentle sample treatment during fractionation. The fractionation mechanism of AF4 is briefly described in this paper but for the interested reader more rigorous descriptions can be found elsewhere [1–3].

The fractionation principle is illustrated in Fig. 1. In brief, the bottom of the AF4-channel (accumulation wall) consists of an ultrafiltration membrane with a given cut-off which allows some of the carrier liquid entering the channel inlet to exit through the accumulation wall (the crossflow). The resulting 'field' gives rise to a convective transport of sample components toward the accumulation wall. The field induced transport is, in turn, counteracted by the Brownian motion of sample components and at steady-state each size fraction in the sample has established a characteristic concentration distribution from the accumulation wall. The characteristic distance from the accumulation wall for a size fraction, at a given crossflow rate, depends on its diffusion coefficient (D). Size fractions with higher diffusion coefficients (smaller hydrodynamic size) are on average situated further away from the accumulation wall than size fractions with lower diffusion coefficients (larger hydrodynamic size). As the longitudinal flow in the channel is laminar, the flow velocity is highest in the center of the channel and decreases as the accumulation wall is approached. Size fractions distributed further away from the accumulation wall experience a higher longitudinal flow velocity and are, thus, transported more rapidly out of the channel than size fractions which are in closer proximity to the accumulation wall. Hence, AF4 fractionates samples based on differences in D (or conversely hydrodynamic size), and sample Ds can be directly determined from elution times [1,4–7].

From measured D values the sample hydrodynamic radius (r_h) is obtained from the Stokes–Einstein equation [8]. The calculation of D from elution times becomes more complicated when utilizing programmed (decaying) crossflow compared to constant crossflow. For rectangular channels the calculation of D (with decaying crossflows) can be performed analytically [9]. However, for trapezoidal channels, which are commercially available today, the calculation of D from elution times needs to be performed numerically [5,6]. Recent findings have shown that good accuracy can be obtained and errors are typically about a few percent [6,7]. However, the error increases when very steep crossflow time gradients are used, i.e., if accurate determination of D is to be obtained, then steeply decaying functions should be avoided.

A range of detection schemes are possible for AF4 allowing specific sample properties of interest to be probed. Detection schemes that have been combined with AF4 include, but are not limited to, multiangle light scattering (MALS), UV, differential refractive index (dRI), fluorescence, light scattering (LS), small angle X-ray scattering (SAXS), and inductively coupled plasma mass spectrometry (ICP-MS) [10–12]. MALS is a powerful detector for the characterization of samples fractionated by AF4. Fitting of MALS data allows the determination of the root mean square radius (r_{rms}), also referred to as the radius of gyration (r_g) for each fraction of the sample [13]. Provided that the refractive index increment with concentration (dn/dc) is known for the analyte, and a concentration detector such as dRI or UV is used, the molar mass (M), or more specifically the weight average molar mass (M_w) of each fraction can also be determined from MALS data.

Combining the obtained detector data with AF4 measured r_h allows for a range of molecular properties to be probed. An interesting conformational parameter that can readily be obtained from AF4 coupled with MALS is the ratio between r_{rms} and r_h which can give valuable information about the conformation and shape of particles and macromolecules as a function of size or molar mass. For a homogenous sphere with a smooth surface (i.e., r_h identical to the geometrical radius) the ratio is equal to 0.775. Typical ratios for macromolecules are between 1 and 2 and ratios >2 generally indicate anisotropy, i.e., elongated conformations. Ratios <0.7 typically represents highly swollen structures ("micro gels") [14]. The ratio can, thus, be used to distinguish between different conformations present within a population. Furthermore, the combination of r_{rms}/r_h together with apparent densities has been proposed as a means of distinguishing between aggregated and non-aggregated structures within a population [15,16].

The apparent density (ρ_{app}) over a size distribution is obtained from M and radii data, utilizing either r_{rms} or r_h as

$$\hat{\rho}_{rms,i} = \frac{M_i}{V(r_{rms})_i \cdot N_A} \cdot \alpha \quad \text{or} \quad \hat{\rho}_{h,i} = \frac{M_i}{V(r_h)_i \cdot N_A} \tag{1}$$

where M_i is the molar mass of fraction i, V_i is the volume of fraction i, N_A is the Avogadro number and α is given by Eq. (2) where r is the geometrical radius of a sphere.

$$\alpha = \frac{V_{\text{sphere}}(r_{rms})}{V_{\text{sphere}}(r)} = \frac{r_{rms}^3}{r^3} = \frac{\left(\sqrt{3/5}\cdot r\right)^3}{r^3} = \left(\frac{3}{5}\right)^{3/2} \tag{2}$$

Although an apparent property, it describes the distribution of mass/volume for each fraction and, thus, provides scaling information. A relationship between the overlap concentration, c^*, in macromolecular solutions has, for instance, been shown for high amylopectin starch [17]. The apparent density is also linked to branching density as highly branched macromolecules are expected to display a higher density than less branched. For instance, glycogen which is an $\alpha(1\rightarrow4)$ glucan with $\alpha(1\rightarrow6)$ linked branches and a degree of branching (DB) of 0.07–0.10 displays considerably higher apparent densities than amylopectin [18], which also is an $\alpha(1\rightarrow4)$ glucan with $\alpha(1\rightarrow6)$ linked branches although with lower DB (approximately 0.05) [19,20].

The purpose of this paper is to illustrate some examples of how AF4 can be utilized for the characterization of complex colloidal dispersions and how the method can provide a range of valuable data over highly polydisperse size distributions.

2. Protein and polysaccharide aggregates

Many natural macromolecules are prone to form aggregates in solution. Macromolecular aggregates can play an important role in many applications in the life sciences.

2.1. Casein micelles

Casein micelles are naturally occurring colloidal protein aggregates in milk that play a large role in the properties and rheology of many dairy products such as cheese and yogurt. The size distribution and structure have been shown to be of great importance for casein micelle aggregation [15,21,22]. The size and conformational properties of casein micelles have been investigated with AF4 coupled with MALS [15]. In this study it was found that a majority of the casein micelles had conformational properties close to that of a solid sphere, as would be expected (Fig. 2). However, a larger size but lower concentration fraction had drastically different properties corresponding to non-isotropic conformations with lower apparent densities than the smaller fraction. The results pointed in the direction of aggregated structures consisting of several individual casein micelles. Through comparison with modeling results it could be concluded that the aggregates should consist of

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