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Impact of asymmetrical flow field-flow fractionation on protein aggregates stability

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ABSTRACT

The impact of asymmetrical flow field-flow fractionation (AF4) on protein aggregate species is investigated with the aid of multiangle light scattering (MALS) and dynamic light scattering (DLS). The experimental parameters probed in this study include aggregate stability in different carrier liquids, shear stress (related to sample injection), sample concentration (during AF4 focusing), and sample dilution (during separation). Two anti-streptavidin (anti-SA) IgG1 samples composed of low and high molar mass (M) aggregates are subjected to different AF4 conditions. Aggregates suspended and separated in phosphate buffer are observed to dissociate almost entirely to monomer. However, aggregates in citric acid buffer are partially stable with dissociation to 25% and 5% monomer for the low and high M samples, respectively. These results demonstrate that different carrier liquids change the aggregate stability and low M aggregates can behave differently than their larger counterparts. Increasing the duration of the AF4 focusing step showed no significant changes in the percent monomer, percent aggregates, or the average Ms in either sample. Syringe-induced shear related to sample injection resulted in an increase in hydrodynamic diameter (d_h) as measured by batch mode DLS. Finally, calculations showed that dilution during AF4 separation is significantly lower than in size exclusion chromatography with dilution occurring mainly at the AF4 channel outlet and not during the separation. This has important ramifications when analyzing aggregates that rapidly dissociate (<~2 s) upon dilution as the size calculated by AF4 theory may be more accurate than that measured by online DLS. Experimentally, the d_hs determined by online DLS generally agreed with AF4 theory except for the more well retained larger aggregates for which DLS showed smaller sizes. These results highlight the importance of using AF4 retention theory to understand the impacts of dilution on analytes.

1. Introduction

Development of protein therapeutics is challenging due to the propensity of proteins to form aggregates that may result in reduced efficacy or immunogenicity [1,2]. These aggregates often span a wide range of sizes and thus, separation methods are critical for assessing their size and concentration distributions in therapeutic formulations [3]. Size exclusion chromatography (SEC) is commonly used for the separation and characterization of protein aggregates. However, the potential for sample adsorption to the column packing, shear degradation, high column pressures, carrier fluid additives, and low separation selectivity for large species (>10^5g/mol) limit SEC's applicability [4–9].

Asymmetrical flow field-flow fractionation (AF4) is a complementary method to SEC because its open channel leads to lower shear rates and applicability to larger size analytes [10]. As shown in Fig. 1, the AF4 channel has a trapezoidal shape with one wall formed by a semi-permeable membrane. Fluid flowing into the channel inlet is divided into a crossflow $\left(\dot{V}_c\right)$ that leaves through the membrane wall and a channel flow $\left(\dot{V}_{out}\right)$ that exits through the channel outlet. The separation is based on establishing a parabolic channel flow profile down the axial channel length, a perpendicular cross flow that transports all species to the accumulation wall, and differences in the translational diffusion of sample components that positions each component in different velocity streamlines of the parabolic flow profile. In the normal separation mode, smaller analytes elute first and the diffusion coefficient (D) can be determined from retention time (t_r). The Stokes-Einstein equation, which assumes a spherical shape, can be used to relate D to a hydrodynamic diameter (d_h) [11]. Equation (1) shows the relationship between t_r and D for retention ratios <0.2

$$t_r = \frac{w^2}{6D} ln\left(1 + \frac{\dot{V}_c}{\dot{V}_{out}}\right) \quad (1)$$

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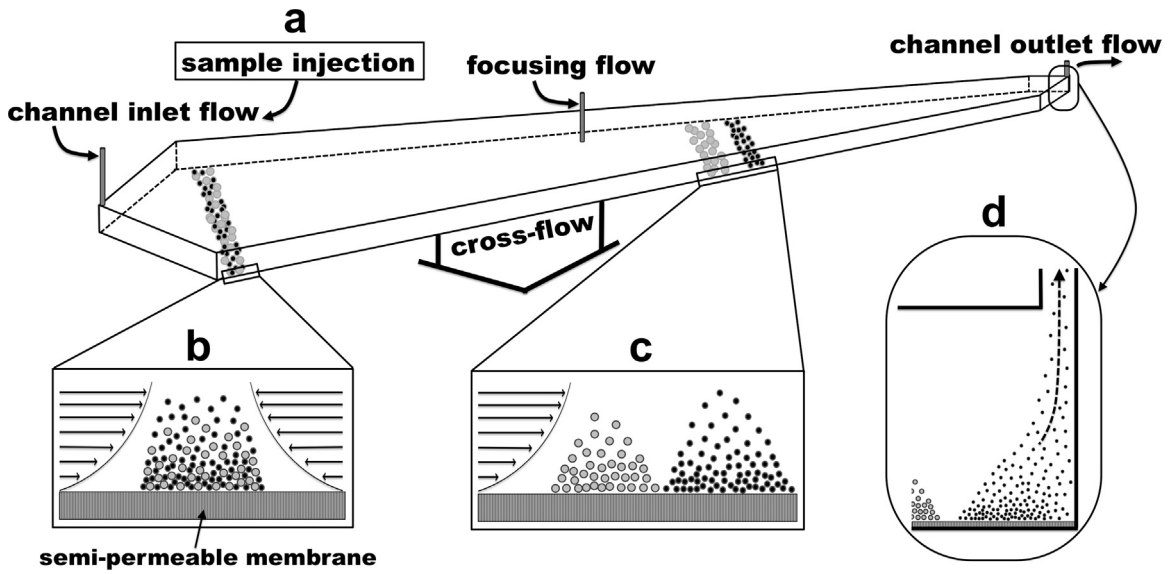

Fig. 1. Schematic of the AF4 channel showing processes during analysis.

where w is the channel thickness [12]. In many cases, AF4 also allows greater flexibility in carrier fluid choice compared to SEC [13] and is able to separate over a wide size range (0.01–>1 μm) with good selectivity (>0.5) [14]. These advantages are at the heart of many publications that provide insights into the formation and dissociation of biological and synthetic complexes and aggregates [15–18]. AF4 determination of dissociation constants (K_ds) has allowed the true stoichiometric binding of protein–protein [15] and DNA-protein [16] complexes to be assessed in solution at <μM affinities. Detection of weakly bound aggregate species can also be achieved by adjusting AF4 conditions [17]. The dissociation of polymer complexes and micelles loaded with fluorescent dyes or nanoparticles has been monitored by AF4 [18,19]. For example, by monitoring the decrease in fluorescence of dye loaded polymer micelles, the stability of the complexes in human plasma could be determined.

Despite these successful demonstrations of the advantageous characteristics of AF4 [20–22], there have been concerns that several steps during the AF4 process may affect delicate or weakly bound protein aggregates species [3,9]. These steps include 1) sample introduction using a syringe, 2) sample focusing and the resulting concentration effect, and 3) separation and the associated shear stress and sample dilution. Steps 1 and 3 are also common to SEC. Current understanding of the impact of each step (and the need for additional studies) is described in the following paragraphs.

During sample introduction there is potential for perturbation of the protein aggregate species due to syringe induced shear stress. Effects of shear stress have been well studied for the production of protein therapeutics [23], administration using pre-filled syringes [24], and flow in physiological systems [25] because of the potential to induce protein aggregation. These studies focus on the aggregation of monomer and not on the changes to pre-existing aggregate species which is equally important to monitor because of their effect on efficacy. It has been noted that shear stress alone may not be sufficient to induce aggregation and solid-liquid or liquid-air interfaces also play a significant role [23,26,27]. Syringes with 22 gauge (0.413 mm inner diameter) needles are typically used to load samples into the injection valve (Fig. 1a). Shear experienced during this step can lead to changes in protein aggregate distributions before the sample is even introduced into the channel. To the authors' knowledge, syringe induced shear stress during SEC or AF4 sample introduction has not previously been investigated in the literature.

In the second stage of AF4, sample is loaded into the channel and focused into a narrow band at the beginning of the channel using two opposing flows as demonstrated in Fig. 1b. Fluid exits the channel through the semi-permeable membrane wall thereby simultaneously providing a crossflow 'field'. This sample focusing step occurs prior to fractionation and is unique to AF4. The transport of sample components to the membrane accumulation wall is countered by diffusion away from the wall with each species reaching a different equilibrium height in the channel. The sample concentration is highest near the accumulation wall and decreases exponentially with increasing distance from the accumulation wall [28]. Typically, sample concentrations at the accumulation wall are 10–100 times greater than that of the original sample [29]. The focusing flow is turned off after the focusing step and the sample components are then swept along the length of the channel.

The online concentration that occurs during focusing is beneficial for the analysis of dilute environmental or biological samples. Sample volumes up to 1000 mL can be concentrated up to 10^5 times in a field-flow fractionation (FFF) channel and online concentration has been used to analyze colloids in environmental suspensions, polystyrene latex beads (PSL), and proteins [30–32]. However, the focusing step can also lead to sample aggregation and/or increased membrane interactions if excessively long times are used [33]. The sample focusing step has also been used as an "online incubation" to investigate IgE-aptamer binding [34]. Increasing the focusing time from 3 to 12 min resulted in an increase in the IgE-bound aptamer ratio indicating increased intermolecular interactions. Other studies have investigated sample interactions with the membrane accumulation wall [35], but this subject is beyond the scope of this work and has been summarized elsewhere [36]. The effects of sample concentration are sample dependent and no published studies have probed perturbations in protein aggregate distributions during AF4 focusing.

In the third stage of AF4, analytes can experience shear and dilution as part of the separation process (Fig. 1c). Shear and dilution are inherent to separation techniques and may play a role in dissociation of aggregates [37–39] as previously shown in SEC and cation exchange chromatography studies [9,40–42]. Aggregates can completely dissociate, partially dissociate, or remain intact depending on the timescale of the separation relative to the rate of dissoci-