**Impact of Asymmetrical Flow Field-Flow Fractionation on Protein
Aggregates Stability**

Carmen R. M. Bria, S. Kim Ratanathanawongs Williams*

Laboratory for Advanced Separations Technologies, Department of Chemistry, Colorado School
of Mines, Golden, CO 80401, USA

*Corresponding author:

S. Kim R. Williams

Department of Chemistry

Colorado School of Mines

Golden, CO 80401

USA

Email: kwillia@mines.edu

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 M s 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 ($< \sim 2$ seconds) upon dilution as the size calculated by AF4 theory may be more accurate than that measured by online DLS. Experimentally, the d_h s 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 ($>10^5$ g/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 Figure 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 ($\dot{V}_c$) that leaves through the membrane wall and a channel flow ($\dot{V}_{out}$) 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

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

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 to $>1 \mu\text{m}$) 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_d s) has allowed the true stoichiometric binding of protein-protein[15] and DNA-protein[16] complexes to be assessed in solution at $<\mu\text{M}$ 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 species aggregate 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 for induced protein aggregation. These studies

1 focus on the aggregation of monomer and not on the changes to pre-existing aggregate species
2 which is equally important to monitor because of their effect on efficacy. It has been noted that
3 shear stress alone may not be sufficient to induce aggregation and solid-liquid or liquid-air
4 interfaces also play a significant role.[23,26,27] Syringes with 22 gauge (0.413 mm inner
5 diameter) needles are typically used to load samples into the injection valve (Figure 1a). Shear
6 experienced during this step can lead to changes in protein aggregate distributions before the
7 sample is even introduced into the channel. To the authors' knowledge, syringe induced shear
8 stress during SEC or AF4 sample introduction has not previously been investigated in the
9 literature.

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

21 The online concentration that occurs during focusing is beneficial for the analysis of dilute
22 environmental or biological samples. Sample volumes up to 1000 mL can be concentrated up to
23 10^5 times in an 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 minutes 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 (Figure 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 dissociation.[43] Shear degradation in SEC columns has been reported for protein aggregates[44] and a variety of high M polymers[45] while shear stress during thermal field-flow fractionation (ThFFF) analysis has shown no evidence of polymer degradation.[46,47] Typically, shear rates in FFF channels ($\sim 1\text{--}20\text{ s}^{-1}$) are orders of magnitude lower than in SEC columns ($\sim 1,000\text{--}10,000\text{ s}^{-1}$)[48]. AF4 without focusing and crossflow has been used to study loosely bound aggregates and showed that these species remained intact during analysis.[13,17,49,50] Therefore, the influence of in-channel shear rate is not specifically addressed in this work. The flow velocity not only determines the shear rate, but also sample dilution during separations. In SEC, sample dilution is

often presented as comparable to AF4.[38] However, sample dilution in AF4 is dependent on the separation conditions and channel dimensions and does not necessarily increase along the length of the separation axis[51], as is the case with SEC. This is because the AF4 sample concentration gradient decreases exponentially with increasing distance from the accumulation wall and occupies a small fraction of the overall channel volume. Therefore, while the effects of dilution have been shown for SEC[38], these studies may not be directly comparable to AF4. Another subtle but important difference is that dilution mainly occurs at the channel outlet as the separated analytes traveling in the proximity of the accumulation wall are swept out along with the carrier liquid that occupies the majority of the FFF channel volume (Figure 1d). This final dilution at the channel outlet is on the order of seconds, but merits investigation as rapidly dissociating aggregates may be present in different forms before and after the channel outlet and this aspect has not been previously considered.

The effect of different carrier liquids on AF4 performance in analyzing proteins have been reported[13,52] but, there are no published studies that systematically investigate the impacts of injection shear stress, concentration, and dilution on protein aggregate stability during AF4. Furthermore, few studies exist that examine how aggregate stability changes with size or M . [53] In this work, low and high M heat stressed anti-SA IgG aggregates are prepared in phosphate or citric acid buffer using AF4. The low and high M aggregates are then re-analyzed by AF4-MALS, AF4-DLS and/or batch mode DLS to determine the effects of syringe shear stress, concentration, and dilution on aggregate stability.

2. Materials and Methods

2.1. Chemicals

Purified anti-streptavidin (anti-SA) IgG1 was provided by Amgen (Thousand Oaks, CA) in formulation buffer at ~30 mg/mL. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, Na_2HPO_4 (anhydrous), NaOH, and NaCl are purchased from Fisher Scientific (Fair Lawn, NJ). Citric acid and sodium azide (NaN_3) are purchased from Mallinckrodt (St. Louis, MO) and Sigma Aldrich (St. Louis, MO), respectively.

2.2. Sample and Carrier Fluid Preparation

All carrier fluid buffers are prepared using distilled deionized (DI) 18.2 M Ω water (APS Filtration AquaMax Ultra, Nan Nuys, CA). Phosphate buffer (100 mM, pH 6.25) is prepared by mixing 10.99 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 3.12 g of Na_2HPO_4 (anhydrous), and 0.20 g of NaN_3 in 1.0 L of DI water. Citric acid buffer (10 mM, pH 5.0) with NaCl (500 mM) is prepared by mixing 2.10 g of $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$ and 29.22 g of NaCl in DI water. Solution pH is adjusted using 1 M NaOH. All buffers are filtered using 0.22 μm mixed cellulose ester filters (Millipore, Bedford, MA).

Anti-SA IgG1 aggregates samples are prepared in the following manner. The concentrated protein is diluted to 1 mg/mL in either phosphate buffer (PBS) or citric acid buffer (CA). Aggregation is induced by heat stressing the PBS and CA sample suspensions using a PolyScience 8000 Constant Temperature Circulator (Niles, IL) set at 67°C for 15 minutes or 60°C for 40 minutes, respectively. After heat stress, samples are immediately immersed in an ice bath for 5 minutes and stored at 2-8°C and processed within 12 hours. AF4 is used to produce the high and low *M* anti-SA IgG1 aggregates samples in PBS, termed PBS-A1 and PBS-A2, and aggregate samples in CA, termed CA-A1 and CA-A2, whose stabilities are subsequently studied and reported in this work. AF4 conditions are provided in section 2.3. Aggregates samples produced in this manner are stable for more than 24 hours as confirmed by DLS and are used within 24 hours of preparation.

2.3. Asymmetrical Flow Field-Flow Fractionation (AF4) and Multi-Angle Light Scattering (MALS) or Dynamic Light Scattering (DLS)

Experiments are performed using an AF2000 system (Postnova Analytics, Salt Lake City, UT) coupled with an SPD-20A UV/Vis detector at 280 nm (Shimadzu, Kyoto, Japan) and a multi-angle light scattering (MALS) DAWN EOS (Wyatt Technologies, Santa Barbara, CA). The *M* and polydispersity index (PDI) are calculated using the Berry model in Wyatt Astra VI software (V 6.1.1.17). A 350 μ m spacer with a maximum width of 2 cm, a minimum width of 0.5 cm, and a length of 29.4 cm was used to form the channel. A 30 kDa molecular weight cutoff regenerated cellulose membrane (Microdyn Nadir, Wiesbaden, Germany) was used as the accumulation wall. Aggregate sample preparations and subsequent PBS and CA aggregate sample analyses were run under the conditions shown in Table 1 unless specified otherwise. Separations where AF4 sizes were calculated from retention theory used constant outlet and crossflow rates. A DAWN HELEOS II (Wyatt Technologies, Santa Barbara, CA) with a DLS attachment with a detector angle of 61.1° was used for online determination of diffusion coefficients.

2.4. Sample Recovery

Sample recoveries are calculated from the area under the UV signal from aggregate sample preparation and subsequent aggregate re-analysis fractograms. The UV peak area from each aggregate sample re-analysis is divided by the UV peak area from the sample preparation and normalized by a factor of 1.2 to account for the injection of 1.25 mL of the total 1.50 mL collected. The percent monomer and percent aggregate for each re-analysis is determined using

the UV peak area of the monomer peak or the aggregate peak and then dividing by the total UV peak area of each re-analysis fractogram.

2.5. Batch Mode Dynamic Light Scattering (DLS)

Size measurements of heat stressed anti-SA IgG1 are performed using a Malvern Zetasizer (Worcestershire, UK) at 25°C.

3. Results and Discussion

3.1. Preparation of Low and High Molar Mass Protein Aggregate Samples

Aggregate samples are prepared by heat stressing anti-SA IgG1 as described in the Experimental section. Heat stress has been shown to be an effective method to accelerate the formation of anti-SA IgG1 aggregate species over a wide size range and allow their formation kinetics to be studied.[14,54] Two commonly used buffers, phosphate and citric acid, are selected to create aggregates of different stabilities and allow comparison of aggregates in different AF4 carrier fluids.

The UV fractograms and the M s for the heat stressed anti-SA IgG1 samples in phosphate buffer (PBS) and citric acid (CA) buffer are shown in Figure 2. In both buffers, a large monomer peak with a uniform M of 1.5×10^5 g/mol is observed at ~5 minutes. The PBS preparation fractogram (Figure 2a) also has a broad peak from 6 to 20 minutes with an M ranging from 2.0×10^5 to 1.0×10^7 g/mol that is attributed to aggregate species. The collected PBS-A1 (low M) and PBS-A2 (high M) sample preparations are shown as the red and the blue shaded areas, respectively. The void peak at 1.5 minutes likely contains large ($>1 \mu\text{m}$) material eluting in the steric mode or small fragments of the monomer formed during the heat stress procedure.[55] The M values are not included due to inaccuracy of MALS above ~400 nm.[56] Aggregate samples are also prepared in a pH 5.0, 10 mM citric acid buffer with 500 mM NaCl. This 10 mM CA

buffer was selected because it has been shown to form large and stable anti-SA IgG1 aggregates when subjected to heat stress.[54] Figure 2b shows the preparation of CA-A1 (low M) and CA-A2 (high M) heat stressed anti-SA IgG1 aggregates samples in CA buffer. A void peak at 1.5 minutes, a monomer peak at 5 minutes and a large aggregate peak from 6 to 22 minutes (3×10^5 to 2×10^7 g/mol) are present.

3.2. Effect of Carrier Fluid on Protein Aggregate Stability

The aggregates stability in PBS and CA was probed by re-injecting the prepared aggregate samples (PBS-A1, PBS-A2, CA-A1, and CA-A2) into the AF4 channel. The AF4 carrier fluids selected for this study are matched to the prepared aggregate sample buffers in order to minimize changes in aggregate species during analysis.

AF4 analysis of the monomer fraction collected from ~3 to 6 minutes (Figure 2) showed no change in aggregation state or M (Supporting information Figure S1). However, analysis of PBS-A1 (low M) and PBS-A2 (high M) aggregates samples yielded a single peak at 5 minutes (Figure 3a) that corresponds to anti-SA IgG1 monomer. The lack of a measurable aggregates peak suggests that the anti-SA IgG1 aggregates completely dissociated to monomer upon preparation and analysis in 100 mM phosphate buffer. Unfortunately, the light scattering intensity is too low to accurately calculate monomer M_s .

Fractograms of both CA-A1 and CA-A2 aggregate samples show dissociation to different extents with monomers formed in both cases (Figure 3b). Furthermore, CA-A1 showed more dissociation to monomer and lower order aggregates than CA-A2 as reflected by differences in retention time ranges of the aggregates peak. These results suggest that more aggregation is induced and aggregates are more stable in 10 mM citric acid (with 500 mM NaCl) compared to 100 mM phosphate buffer. This may be due to the higher ionic strength CA buffer having higher

electrostatic screening and thus stronger protein interactions. The pH and the ion composition of the buffer may also influence the protein interactions. The observed increase in M from 6 – 20 minutes for CA-A2 is due to aggregates that range from oligomers to large species $>2 \times 10^6$ g/mol. The measured M s also confirm that smaller aggregates are present and suggest that both CA-A1 and CA-A2 undergo partial dissociation. The significant difference in aggregates behavior between PBS and CA buffers highlights their role in aggregate stability during AF4 analysis.

3.3. Effect of Syringe Shear Stress During Sample Introduction

Syringe injection is typically used to introduce analytes into the sample loop for SEC and AF4 analyses. Shear stress during syringe injection of therapeutic proteins into patients may also induce aggregation.[23,24] Effects of shear stress on the formation of aggregate species from monomer have been most commonly studied due to the implications for administration of therapeutics to patients[57], but the impact of shear on aggregate species is also important because of the impact on efficacy and dosage.

The effect of syringe shear stress on CA-A1 and CA-A2 samples are analyzed by AF4-MALS and batch mode DLS. The samples are stressed by slowly drawing the sample into the injection syringe and then gently displacing the sample into the cuvette used for DLS analysis. The syringe displacement is performed at the same speed as samples that are introduced into the AF4 injection valve sample loop. Syringe stress was performed one time (SG1X) or three times (SG3X). AF4-MALS analysis of CA-A1 and CA-A2 after syringe stress did not show any significant changes in the aggregate amounts or M s (Supporting information Figure S2). Changes in aggregates formed by shear stress may be reversible under the chosen separation conditions or may not be detectable by AF4-MALS. Hence, further characterization of CA-A1 and CA-A2 aggregate samples by batch mode DLS is performed to monitor the aggregate sizes without the

potential influence of AF4. The sample concentrations for both AF4 and DLS analyses are kept constant. The DLS measured z-average diameter for unstressed and syringe stressed CA-A1 and CA-A2 of are shown in Figure 4. The unstressed z-average diameter for CA-A1 (31.2 ± 7.6 nm) is lower than CA-A2 (40.6 ± 0.2 nm) as expected. After syringe stress, the size increases for both samples. However, SG3X of CA-A1 has a significantly larger z-average diameter than SG3X of CA-A2 which suggests that lower M aggregates are more susceptible to aggregation by shear stress. These results emphasize the need to study syringe-induced shear stress during SEC and AF4 analyses.

3.4. Effect of Sample Focusing Time on Aggregates Stability

Sample focusing plays an important role in improving retention and resolution in AF4 separations.[29,33] Ideally, the focusing time is optimized to reduce potential analyte-analyte and analyte-membrane interactions[36] while maintaining separation performance and decreasing analysis times. However, the sample concentration that occurs during the focusing/relaxation step may form or destroy aggregates. Few studies directly examine the influence of protein aggregate concentration during focusing despite concerns that sample concentration may induce aggregation.[38,49,58,59]

Figure 5 shows the UV responses and M s for the CA-A1 and CA-A2 samples at increasing focusing times. There is no apparent change in t_r , relative peak areas, or M when the focusing time is increased from 7.2 to 13.2 minutes for CA-A1 (Figure 5a). The higher void peak areas for shorter focusing times are likely due to incomplete sample relaxation.[28,60] To elaborate, a 1.25 mL sample requires 6.25 minutes to be completely loaded into the AF4 channel when an injection flow rate of 0.2 mL/min is used. Thus, the last part of the sample plug to enter the channel is subjected to the focusing flows for a shorter period of time (~1 min for 7.2 minutes

1 focusing, ~7 minutes for 13.2 minutes focusing) and may not be fully relaxed. Figure 5b also
2 shows an increase in the void peak for CA-A2 as the focusing time decreases because of
3 incomplete relaxation. Fluctuations in the monomer and aggregate peak intensities are primarily
4 due to differences in the prepared CA-A2 sample amounts. However, the sample recoveries for
5 each focusing time are not significantly different and did not impact the results or conclusions
6 drawn.

7 The weight average M of the aggregate peak and the percent monomer and percent
8 aggregates are calculated for each focusing time (Figure 6). As expected, the CA-A1 M_w is lower
9 than CA-A2 for all focusing times (Figure 6a). No significant difference between average M s is
10 observed with increasing focusing time for either aggregates sample. Similarly, no significant
11 difference is observed in the percent monomer with increasing focusing time for either sample
12 (Figure 6b). Increasing the focusing time from 9.2 to 13.2 minutes did not significantly change
13 the percent aggregate present in CA-A1 or CA-A2. However, comparison of a 7.2 minute
14 focusing time to 13.2 minutes showed a significantly different percent aggregates for both
15 fractions. This difference is most likely due to insufficient relaxation and not a change in the
16 aggregate population.

17 In summary, increasing focusing time did not significantly change the average M or percent
18 monomer or percent aggregate for CA-A1 or CA-A2. This suggests that the focusing step alone
19 does not significantly change the heat stressed anti-SA IgG1 aggregates under the separation
20 conditions used in this work. However, aggregates formed under different stress types and
21 solution conditions may show a different susceptibility to dissociation.[55] It is recommended
22 that the potential impacts of focusing be investigated as part of the AF4 method development for
23 individual different proteins, stress treatments, and solution conditions.

3.5. Effect of AF4 Separation on Aggregates Dissociation

During separations of protein aggregates, there is the potential for dissociation in the channel.[38] Table 2 shows a comparison of t_r s, weight average molar mass (M_w), polydispersity index (PDI), and percent monomer and aggregate for the original preparation and after AF4 analysis of CA-A1 and CA-A2 in CA buffer. The t_r s decrease from 8.5 to 7.2 minutes for CA-A1 and from 11.5 to 10.0 minutes for CA-A2. The shift toward lower t_r s and M_w s upon reinjection suggests that aggregates are partially reversible upon AF4 separation and dissociate to smaller aggregate species. The increase in the PDIs for both aggregate samples confirms the presence of a wider aggregates size distribution. Sample recoveries were ~70% for CA-A1 and CA-A2. The similar recoveries for both low and high M samples suggest that there is no size-dependent sample loss and that sample loss does not affect the conclusions. Both sample preparations should be composed entirely of aggregate species, but after AF4 analysis, CA-A1 and CA-A2 contain 25.0% and 4.5% monomer, respectively. The significantly higher percent monomer in CA-A1 suggests that smaller aggregates are more easily dissociated to monomer than the larger aggregates in CA-A2. However, larger aggregates present in CA-A2 are also partially reversible yielding primarily lower M aggregate species rather than monomeric species. These results demonstrate the ability of a separation technique such as AF4 to provide insights into the different behavior of low and high M protein aggregates. The observed aggregates behavior during separation also leads to the following more detailed analysis of dilution in an AF4 channel.

Sample zone broadening occurs in both FFF and chromatography, but the separation processes and column dimensions are distinctly different leading to different amounts of sample dilution in each technique. Figure 7a shows the axis notations and symbols used in describing an

FFF channel. The sample forms a steady state concentration distribution across the channel thickness (w) with the highest sample concentration at the accumulation wall and the center of gravity at the mean layer thickness (l). The concentration decreases exponentially with increasing distance away from the accumulation wall, but on average the sample is typically concentrated within a distance of ~ 1 - $10\ \mu\text{m}$ from the accumulation wall.[29] This represents only $\sim 3\%$ of the channel thickness or $\sim 6\%$ of the channel volume. Near the beginning of the channel, nonequilibrium is the major zone spreading mechanism.[61] However, the trapezoidal shape of the AF4 channel breadth (wide at the inlet and narrow at the outlet) can actually result in a concentration increase as the sample nears the channel outlet depending on the channel dimensions and the flow conditions used.[62]

Figure 7b shows the calculated concentration at the zone center at each position, z , along the channel length, L , for a $100\ \mu\text{L}$ injection of a $1\ \text{mg/mL}$ anti-SA IgG1 sample and a $27.6\ \text{cm}$ long channel. Calculations are performed as previously shown by Litzén.[51] The change in concentration is primarily a function of changes in nonequilibrium band broadening and the narrowing of the channel breadth from the inlet to outlet. The sample concentration at the accumulation wall (c_∞) initially decreases to $\sim 0.75\ \text{mg/mL}$ and then increases to $\sim 1.25\ \text{mg/mL}$ at the end of the channel ($z = L$), just before the sample exits the channel. The concentration at the mean equilibrium layer thickness (c_l), calculated for a retention ratio of 0.2 , represents the average channel height that analytes experience during separation. The c_l ranges from $\sim 0.25\ \text{mg/mL}$ in the first half of the channel to $\sim 0.5\ \text{mg/mL}$ at the end of the channel. These c_l values represent an average sample dilution of ~ 2 - $4X$ during the separation. The maximum dilution occurs at $z \sim 10\ \text{cm}$ with an average sample concentration of $\sim 0.25\ \text{mg/mL}$. The contribution of nonequilibrium band broadening can also be determined using the diffusion coefficient of the

analyte, the mean carrier fluid velocity, and the nonequilibrium coefficient (a function of sample retention) along L from the focusing position to the end of the channel ($z=4$ to $z=27.6$ cm).[29] Figure 7c shows the concentration distribution as a function of distance from the accumulation wall and nonequilibrium band broadening σ (where $\sigma = 0$ is the center of the sample band). By integrating the area under each concentration distribution at each channel height, the percent of the sample that experiences a certain concentration during separation can be determined. Overall, ~85% of the sample is present below $2l$ (or $19\text{ }\mu\text{m}$ for the conditions used) with an average concentration of 0.1 to 1.25 mg/mL.

In both AF4 and SEC, the separation results in sample dilution through band broadening dispersion, but in contrast to AF4 there is no concentration mechanism in SEC. This results in sample dilutions of 20X to 100X in SEC depending on the column and separation conditions compared to the 2X to 4X dilution experienced during AF4. The majority of the dilution in AF4 occurs at the channel outlet when the separated sample zones, near the accumulation wall, mix with the carrier fluid in the ‘top’ part of the channel (Figure 1d) just prior to eluting. The outlet sample concentration ($\bar{c}$) can be calculated using Equation 2[63]

$$\bar{c} = \frac{m}{4V_r} (L/\bar{H})^{1/2} \quad \text{Eqn 2}$$

where m is the injected sample mass, V_r is the product of t_r and $\dot{V}_{out}$ (the volumetric flow rate at the outlet), and $\bar{H}$ is the observed band broadening (determined experimentally). Based on a 100 μL injection of a 1 mg/mL anti-SA IgG1, the concentration as the sample exits the channel is 0.052 mg/mL, a ~20X dilution. Using a typical volumetric flow rate of 0.5 mL/min, it takes ~1 to 2 seconds for the sample to flow from the channel outlet to the detector. If dissociation occurs in >~2 seconds, the aggregate species flowing through the detector will be the same as that at $z = L$ in the AF4 channel. However, if dissociation occurs under ~2 seconds, a smaller analyte will be

measured by the online detector. Since the bulk of the dilution (and subsequent dissociation) occurs at the channel outlet, the measured retention time is relatively unchanged. This means that the measured AF4 $t_{r,s}$ may provide more accurate sizes than online detectors for samples that undergo rapid dissociation upon dilution. This is an important point to consider particularly when the aggregates size calculated using AF4 theory is larger than that measured by online MALS or DLS (assuming no analyte-membrane interactions).

The hydrodynamic diameter (d_h) determined by online DLS and AF4 theory are compared to examine the effects of changes in sample dilution during the separation. The average sizes determined by DLS and AF4 are z-average and number average, respectively. If the common assumption that analytes are monodisperse for each measured slice is used, then the DLS and AF4 sizes are expected to be equivalent. Figure 8 shows the AF4-DLS fractograms of heat stressed anti-SA IgG1 obtained using different absolute crossflow and channel outlet flow rates while holding the ratio of $\dot{V}_c/\dot{V}_{out}$ constant at 2. Under such conditions, sample components are expected to have the same $t_{r,s}$, but increased sample dilution. Plotting the fractograms in terms of retention volume instead of retention time highlights the effect of changing absolute flow rates. At a $\dot{V}_c/\dot{V}_{out}$ of 0.5/0.25, the decreased resolution causes a deviation of the d_h values between online DLS measurements and AF4 theory. Lower resolution is expected at lower absolute flow rates[63] and the decrease in resolution results in coelution of aggregates with large analytes that subsequently influences the sizes determined by online DLS. Good agreement between DLS and AF4 theory sizes is observed for $\dot{V}_c/\dot{V}_{out}$ of 1.0/0.5 and 1.5/0.75 although the higher dilution at the outlet of the latter results in a noisier DLS signal. Interestingly, the DLS sizes become lower than AF4 theory sizes at higher retention volumes for all flow rates investigated. This may be due to the dissociation of larger, longer retained aggregates due to dilution at the outlet. Potential

dissociation of aggregates upon dilution at the channel outlet highlights the importance of performing flow rate experiments such as those described here and comparing sizes derived from AF4 theory and online DLS. As explained earlier, shear rates in AF4 are low and published studies have shown no flow-induced dissociation. It should also be noted that the increased $\dot{V}_{out}$ from 0.25 to 0.75 mL/min corresponds to a small increase in the shear rate from ~ 4 to 12 s^{-1} . Hence, the effect of shear rate is not specifically examined in this work and the observed effects have been mainly attributed to dilution.

4. Conclusion

The effects of carrier fluid, syringe shear stress, focusing/concentration, and dilution on aggregate stability is investigated for aggregates samples of two different M s and sizes. Carrier fluid had a significant impact on aggregates stability with nearly complete dissociation observed in PBS and partial dissociation in CA buffer. Also, low M aggregates in CA buffer were more likely to dissociate to monomer than high M species indicating size-dependent aggregates stability. Syringe shear stress associated with sample loading altered aggregate size distributions as measured by batch mode DLS, although no changes were observable by AF4-MALS. Similarly, sample concentration as a result of the focusing step did not appear to change the aggregate distributions. Theoretical calculations showed that sample dilution *in* the AF4 channel is $\sim 2\text{-}4\text{X}$ and that the majority of the AF4 dilution ($\sim 20\text{X}$) occurred at the channel outlet as the separated sample zones eluted. Since the difference in time at the end of the trapezoidal channel and the channel outlet is small, AF4 retention theory may provide more accurate size information than online light scattering detectors, particularly for aggregates that rapidly dissociate upon dilution. This highlights the importance of comparing AF4 retention theory sizes with those measured by orthogonal methods (such as light scattering detectors).

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Figure Captions

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

Figure 2. Fractograms (line) and M_s (symbols) showing the preparation of a) PBS-A1 and PBS-A2 and b) CA-A1 and CA-A2 samples of heat stressed anti-SA IgG1 aggregates. b) Fractograms for the analysis of the PBS-A1 (red line) and PBS-A2 (blue line) samples.

Figure 3. a) Fractograms (line) and M_s (symbols) for the analysis of a) PBS-A1 (red line) and PBS-A2 (blue line) and b) CA-A1 (red line and symbols) and CA-A2 (blue line and symbols) samples. The UV signals are scaled by factors of 8X and 4X for the PBS and CA samples, respectively.

Figure 4. a) z-average diameters determined by batch mode DLS for CA-A1 and CA-A2 before and after syringe stress. Error bars represent standard deviations with $n \geq 3$.

Figure 5. Fractograms (lines) and M_s (symbols) for analysis of a) CA-A1 and b) CA-A2 with increasing focusing times.

Figure 6. a) The average M_s and b) the percent monomer and percent aggregate for the reinjection of CA-A1 and CA-A2 with focusing time. The void peak is not included in the percent monomer and percent aggregate calculations. Error bars are standard deviations ($n \geq 3$) and are smaller than the symbols if not visually apparent.

Figure 7. a) Diagram of the channel geometry. b) Calculated concentration of anti-SA monomer along the channel and c) sample zone concentration distributions at $z=16$ cm and different distances from the accumulation wall. The letter l represents the mean equilibrium layer thickness.

Figure 8. Fractograms for heat stressed anti-SA IgG1. The absolute $\dot{V}_c/\dot{V}_{out}$ in mL/min for the black, red, and blue traces are 0.5/0.25, 1.0/0.5 and 1.5/0.75, respectively. Filled symbols and closed symbols represent d_{hs} determined by online DLS and AF4 theory, respectively.

Figure1

Figure2a

Figure2b

Figure3a

Figure3b

Figure4

Figure5a

Figure5b

Figure6a

Figure6b

Figure7a

Figure7b

Figure7c

Figure8

Table 2. Comparison of CA-A1 and CA-A2 Original and Re-analyzed Samples

	CA-A1		CA-A2	
	Original	Re-analysis	Original	Re-analysis
t_r^* (min)	8.5	7.2 ± 0.1	11.5	10.0 ± 0.1
M_w ($\times 10^6$ g/mol)	1.35 ± 0.08	1.11 ± 0.10	4.77 ± 0.42	3.01 ± 0.11
PDI	1.14 ± 0.01	1.30 ± 0.05	1.12 ± 0.01	1.31 ± 0.06
Monomer (%)**	-	25.0 ± 2.1	-	4.5 ± 0.3
Aggregate (%)**	-	68 ± 4	-	91 ± 2

Uncertainties represent 95% confidence intervals.
* t_r s taken at the median of the original fraction.
** Percentages do not add up to 100% because material eluting in the void peak is not included.

Table 1. AF4 Conditions for the Preparation and Re-analysis of Aggregates Samples

	Sample Loop Volume	Focusing Flow Rates (inlet; focus)	Focusing Time	Separation Flow Rates (crossflow; outlet)
Aggregate Sample Preparation	100 µL	0.2 mL/min; 1.3 mL/min	3 min	1.0 mL/min to 0.1 mL/min over 10 minutes; 0.5 mL/min
Aggregate Sample Re-analysis	1.25 mL	0.2 mL/min; 1.3 mL/min	7.2, 9.2, 11.2, and 13.2 min	1.0 mL/min to 0.1 mL/min over 10 minutes; 0.5 mL/min