DEVELOPMENT OF ASYMMETRICAL FLOW FIELD-FLOW FRACTIONATION FOR THE CHARACTERIZATION OF PROTEINS, PROTEIN AGGREGATION, AND NANOPARTICLES

by

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A thesis submitted to the Faculty and the Board of Trustees of the Colorado School of Mines in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Applied Chemistry).

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Field-flow fractionation (FFF) is a family of analytical techniques used to characterize macromolecules and particles from $\sim 1$ nm to $>1$ $\mu$m. Its versatility has allowed a number of analytical challenges to be addressed, but several limitations still exist. Advances in asymmetrical flow field-flow fractionation (AF4) coupled with UV-Vis, multiangle light scattering (MALS), and/or dynamic light scattering (DLS) were made to overcome current limitations in the characterization of proteins, protein aggregates, and nanoparticles. Formation of protein aggregates in protein therapeutics is a major concern due to reduced drug efficacy and potential immunogenicity. A lack of reliable analytical methods that cover the submicron (0.1-1 $\mu$m) size range has been a major challenge for protein aggregate characterization. The development of a simple AF4 method with good size selectivity ($>0.5$) from 1 nm to 1 $\mu$m allowed the formation of submicron aggregates to be fitted by the Lumry-Eyring Nucleated Polymerization (LENP) model for the first time. A comparison of aggregation kinetics determined by AF4 and the LENP model before and after centrifugation of aggregate samples showed that this common sample preparation step prior to sized exclusion chromatography (SEC) might influence the experimentally observed kinetic mechanism. The results suggest that AF4 is able to provide more reliable kinetic data for aggregates up to and larger than 100 nm that may not be easily characterized by SEC.

AF4 provides a wealth of information including analyte size distributions, but this information can be influenced by the inherent dilution that occurs during separations, especially for weakly bound protein aggregates. Protein aggregate stability at each stage of the AF4 analysis was studied. The choice of carrier fluid played a significant role in aggregate stability while the AF4 focusing step did not have significant impact on aggregate populations. Calculations showed that sample dilution is significantly lower in AF4 than in SEC and that dilution occurred primarily at the channel outlet (not during the separation). This suggests
that sizes from AF4 theory may be more accurate than those from online light scattering
detectors because rapidly dissociating species may be altered upon dilution at the channel
outlet. Understanding aggregate behavior during AF4 is critically important, not only for
protein aggregates, but also for polymer and nanoparticle supramolecular complexes that
may be altered by analysis.

A major challenge that impacts AF4 analyses of samples from nanoparticles to polymers
to proteins is unwanted analyte-membrane interactions. These interactions can potentially
be reduced by modifying the membrane surface, but modification of AF4 membranes has
been restricted by two key challenges: 1) large membrane areas (∼90 cm²) must be modi-
fied and 2) the membrane surface must remain flat and semi-permeable. The development
of a method to modify membranes for AF4 analysis has provided the foundation to over-
come these challenges. The novel channel reactor developed in this work allows large, flat
membrane surfaces to be grafted with polymer brushes while simultaneously reducing the
reagent amounts required. Poly(N-isopropylacrylamide) brushes were successfully grafted
from membranes resulting in thermo-responsive behavior to provide control over the mem-
brane hydrophobicity. The modified membranes were then used for AF4 analysis of IVIg
and BSA proteins. This method provides the necessary groundwork for addressing perhaps
the most significant limitation of AF4, analyte-membrane interactions.

Finally, the design and optimization of semi-preparative scale AF4 channels has pushed
the boundaries of the sample amounts that can be separated in a single run. Separations of
protein aggregates, biological particles, and other nanoparticles are currently limited to small
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Nanotechnology, polymer science, and biotechnology research have expanded rapidly over
the last two decades. New nanomaterials are revolutionizing catalysis and energy production,
polymers are ubiquitous in modern day life, and nano-based therapeutics can now treat
previously untreatable diseases. However, these nano-scale materials are often polydisperse in
size and/or composition and may form complexes and/or aggregates. Separation techniques
to purify and analyze these complex materials are highly desired to understand the impacts
of polydispersity and aggregation on their properties and performance.

In particular, the characterization of protein aggregates is a major analytical challenge
during the development and production of protein-based therapeutics. The formation of ag-
ggregates in therapeutic protein formulations pose a significant risk to patients due to reduced
efficacy and potential for unwanted immunological responses.[1–4] Aggregation can occur
during manufacturing, transportation, storage, and administration of protein therapeutics.[5,
6] Non-native aggregation refers to the association of partially unfolded protein structures.
External factors including heat or chemical stress, changes in pH or ionic strength, and
exposure to liquid-air or liquid-solid interfaces can promote protein unfolding and lead to
the formation of aggregates. This wide range of stress factors leads to aggregates with a
wide range of physicochemical properties that influence their potential immunogenicity.[7–9]
The stability and size of these complex species is determined by both protein properties and
solution properties. This results in species that may be reversible or irreversible and span
sizes from nanometers to centimeters. The potential for dissociation and the large size range
makes the characterization of aggregates challenging. The Food and Drug Administration
often requires multiple orthogonal methods be used for size analysis.[10] The submicron (0.1
µm to 1 µm) size range is particularly challenging due to the lack of analytical methods.[2]
1.1 Analytical Techniques for Protein Aggregate Characterization

The wide size range of aggregate species necessitates the use of multiple analytical techniques. Batch mode (non-separation-based) techniques are used to obtain an average size for a given sample. However, the high polydispersity of many aggregate samples require separation-based methods to provide more detailed information about a potentially dynamic system. Furthermore, many of the current non-separation and separation-based analytical techniques are specific to a particular range of sizes (Figure 1.1). Possibly one of the most widespread techniques used to determine size of particles from \(\sim 1\) nm to \(\sim 1\) \(\mu\)m is dynamic light scattering (DLS). This technique measures fluctuations in light scattering of the sample and mathematically correlates the fluctuations back to the diffusion coefficient \((D)\) of the analytes in solution. The main advantages of DLS are that with relatively little sample preparation, measurements can be made in a matter of minutes and particle size distributions can be determined to provide information about polydispersity. However, well known limitations also exist.[11] Light scattering is inherently more sensitive to large size particles. This means that a small amount of large particles can dramatically influence the measurement resulting in erroneously high sizes. In turn, this results in the size resolution of DLS where particle sizes must differ by a factor of at least 3 to be resolved. Finally, commercially available instruments rely on proprietary algorithms to determine sizes and the assumptions in the calculation (including spherical particles) may not be true or fully understood.

Nanoparticle tracking analysis (NTA) operates on a similar principle and over a similar size range (\(\sim 40\) nm to \(\sim 1\) \(\mu\)m) to DLS.[11, 12] In NTA, light scattered by particles is monitored with a CCD camera. The size of the particles is determined by measuring the Brownian motion of individual particles in solution to determine the diffusion coefficient \((D)\) of each particle. The \(D\) can then be use to calculate a hydrodynamic diameter \((d_h)\) assuming a spherical geometry. Monitoring individual particles reduces the bias towards larger sizes in comparison to DLS and improves the size resolution. However, scattering from large particles can still mask smaller particles leading to biases toward larger sizes.
Figure 1.1: Commonly used analytical techniques used to characterize the size of protein aggregates. FFF: field-flow fractionation; SEC: size exclusion chromatography; AUC: analytical ultracentrifugation; DLS: dynamic light scattering; NTA: nanoparticle tracking analysis; RMM: resonance mass measurements; TEM: transmission electron microscopy.

Additionally, NTA has a lower size limit of ∼40 nm which means most protein monomers are undetectable. Other techniques such as resonance mass measurement (RMM), flow cytometry, and Coulter counter have also been used to characterize particles from ∼0.5 µm to >1 µm in size.[13] These techniques bridge the size range around 1 µm, the maximum size for diffusion-based techniques (DLS and NTA) and the minimum size for optical microscopy based techniques.[14] However, they are unable to detect monomeric species and aggregates <0.5 µm.

Flow imaging has been widely adopted over the past few years to quantify the size, morphology, and number of particles from 1-10 µm.[15, 16] A CCD camera is used to capture images from an optical microscope as the solution of particles is flowed through the field of view. This technique can distinguish between particles composed of different materials and is able to give some information about the particle morphology. However, because the technique is based on optical microscopy, the size range is limited to particles >1 µm.[17]
Transmission electron microscopy (TEM) can be used to characterize the size and morphology of aggregates below 1 µm. However, TEM is commonly used in combination with other analytical techniques because of its well-known limitations.[18] The biggest drawback of a typical TEM measurement is the drying of samples for analysis that can lead to particle aggregation. Additionally, for many biological samples, stains must be used to provide sufficient contrast.

Size exclusion chromatography (SEC) is one of the most common size separation techniques used to characterize proteins, polymers, and nanoparticles. The separation takes place in a column packed with a porous material. Analytes dissolved in a carrier move in and out the pores as they pass through the column. Small analytes can access more of the pore volume than larger analytes and elute later. SEC is the workhorse of protein and polymer separations.[19] The size range of SEC is typically 104 to 106 kDa for polymers and proteins and <200 nm in diameter for particles and aggregates.[2] The upper molecular weight (MW) size range can be extended, but shear stress during the separation can result in the destruction of weakly bound complexes (like protein aggregates) and large or highly branched analytes.[20]

Concerns that SEC may alter sample properties during analysis have led to the use of orthogonal methods including analytical ultracentrifugation (AUC) and field-flow fractionation (FFF).[2, 14, 21] Particles up to ~100 nm can be characterized by AUC and there is relatively low sample dilution during analysis (~10 to 20X dilution) making it a valuable tool for characterizing weakly bound species that are prone to dissociation.[22] This also allows dissociation constants to be determined. However, the technique suffers from low throughput and complicated data analysis. Analyses can take days to complete and there is no possibility for collection of fractions after the separation.

Field-flow fractionation (FFF) is a family of separation techniques for proteins, polymers, and nanoparticles that addresses many of SECs limitations.[23] In FFF, an open channel is used to separate analytes based on their size, mass, and/or composition depending on the
selected field. Retention theory of FFF is generally well understood which allows analyte properties to be determined based on their retention time ($t_r$). The open channel reduces the shear stress during the separation and highly branched polymers, weakly bound proteins, and large aggregates can be analyzed.\textsuperscript{[24–28]} The lack of packing material also limits the amount of sample adsorption during analysis. Furthermore, fractions can be collected after analytes have been separated providing more narrowly dispersed sample populations for additional analyses. Knowledge of the FFF separation mechanism and retention theory is important for understanding its advantages. The theory of the FFF separation and the advantages and limitations in relation are summarized in the next sections.

1.2 Field-Flow Fractionation Mechanism and Theory

The FFF separation is implemented in a thin, open channel (Figure 1.2). A parabolic flow is formed across the channel thickness, $w$, and moves along the separation axis with the slowest velocities at the walls and the highest velocities in the center of the channel. A field is applied perpendicularly to the separation axis forcing analytes towards the accumulation wall. This field-induced transport is counteracted by analyte diffusion away from the accumulation wall resulting in an exponential concentration gradient with the highest concentration at the wall. This mechanism is called the normal mode of FFF where small analytes with higher diffusion coefficients ($D$) elute before larger analytes. The retention ratio ($R$) is the experimentally relevant quantity in FFF given by Equation 1.1

$$R = \frac{t_0}{t_r}$$

where $t_r$ is the elution time of the retained peak and $t_0$ is the elution time of the unretained peak (also called the void time).

Physically, the $R$ represents the balance of the applied force field ($F$) and the opposing sample diffusion described by the diffusion coefficient ($D$). At equilibrium, there is no net flux of analytes in either direction and the analyte concentration ($c(x)$) has an exponential...
distribution described by Equation 1.2

$$c(x) = c_0 \exp \left( -\frac{xU_x}{D} \right)$$  \hspace{1cm} (1.2)

where \( x \) is the position in the \( w \) dimension, \( c_0 \) is the concentration at the accumulation wall \((x = 0)\), and the velocity of analytes towards the accumulation wall, \( U_x \), is \( F \) divided by the friction coefficient. An alternate and convenient form of \( F \) can be written as

$$F = U_x f = kT \frac{U_x}{D}$$  \hspace{1cm} (1.3)

where \( k \) is Boltzmann’s constant and \( T \) is the temperature. This form can be used to describe the force on the analyte cloud for FFF techniques.

The mean layer thickness \((l)\) is the distance from the accumulation wall to the center of mass of the analyte cloud and is also defined as the ratio of thermal energy to the applied force \( l = kTF \). A dimensionless retention parameter, \( \lambda = lw \), represents the compression of
the analyte zone and is related to F for FFF techniques in general by Equation 1.4

$$\lambda = \frac{kT}{Fw} \quad (1.4)$$

This parameter physically represents the distance of the sample from the accumulation wall, it is related to the interaction of the sample and the applied force, and can be related to the experimental quantity $R$ defined as

$$R = \frac{v_{zone}}{\langle v \rangle} = \frac{\langle c(x)v(x) \rangle}{\langle c(x) \rangle \langle v(x) \rangle} \quad (1.5)$$

where $v_{zone}$ is the average velocity of the analyte zone, $\langle v \rangle$ is the average flow velocity in the channel. The flow velocity profile across $w$, $v(x)$, is given by Equation 1.6.

$$v(x) = 6\langle v \rangle \left[ \frac{x}{w} - \left( \frac{x}{w} \right)^2 \right] \quad (1.6)$$

Using Eq. 1.2 and Eq. 1.6, Eq. 1.5 can be integrated analytically to give

$$R = 6\lambda \coth \left( \frac{1}{2\lambda} \right) - 12\lambda^2 \quad (1.7)$$

When $\lambda$ values are <0.02, $R$ values with errors of <5% can be calculated using the approximation $R = 6\lambda$. Finally, this approximation can be related to physical and experimental quantities by Equation 1.8.

$$R = \frac{t^0}{t_r} = 6\lambda = \frac{6kT}{Fw} \quad (1.8)$$

There are a number of fields that can be used to fractionate analytes in FFF including thermal, sedimentation, flow, electrical, magnetic, etc. Of these fields, thermal, sedimentation, and flow field instruments are commercially available. Calvin Giddings, the inventor of FFF, demonstrated the technique in 1966 by separating polymers using thermal field-flow fractionation (ThFFF).[29] Heating the top block and cooling the bottom block of the channel establishes the thermal field. In SdFFF, the channel is placed in a centrifuge and particles experience a centrifugal force.

The most widely used FFF technique is asymmetrical flow field-flow fractionation (AF4). In AF4, one wall is permeable to the carrier fluid, but not to the analyte. Fluid leaving
through the semi-permeable wall creates a cross-flow field driving force on the analytes given by Equation 1.9

$$F = f|U| = 3\pi \eta |U|d$$ \hspace{1cm} (1.9)

where $f$ is the friction coefficient, $U$ is the cross-flow velocity at the accumulation wall, and $\eta$ is the viscosity. The surface area of the accumulation wall is equal to the product of the geometric channel volume, $V_0$, and $w$ so $U$ can be written:

$$U = \frac{\dot{V}_c w}{V_0}$$ \hspace{1cm} (1.10)

where $\dot{V}_c$ is the cross-flow rate. The combination of Eqs. 1.8, 1.9, and 1.10 gives:

$$t_r = \frac{\pi \eta \dot{V}_c w^2}{2kTV_0} d_h$$ \hspace{1cm} (1.11)

The use of a flow field results in a direct correlation between $t_r$ and $d_h$.

Until now only the normal mode of FFF has been discussed. However, as the analyte size increase their diffusion decreases and eventually their Brownian motion becomes negligible. Analytes are forced towards the accumulation wall, but the negligible diffusion means that the analytes center of mass in the channel is equal to its radius. In this so called steric mode of FFF, larger analytes protrude further into the channel, experience higher channel flow velocities, and elute before smaller analytes. The transition between the normal and steric modes of FFF can be described by the slightly retention ratio in Equation 1.12

$$R = 6\gamma \alpha + 6\lambda$$ \hspace{1cm} (1.12)

where $\gamma$ is the drag factor and $\alpha$ is the ratio of the particle radius, $a$, to $w$ ($\alpha = a/w$). As particle size increases, $\lambda$ approaches zero until the $6\lambda$ term describing the normal mode becomes zero. Conversely, increasing particle size leads to large $\alpha$ values and indicates a shift into the steric mode elution mechanism.
1.3 Asymmetrical Flow Field-Flow Fractionation Advantages, Limitations, and New Advances

AF4 has become by far the most commonly used FFF technique in the past decade because the flow field is felt universally by all analytes and it offers a separation based entirely of $D$ leading to size based separations. In particular, AF4 has become an invaluable technique for the separation and characterization of biological based analytes.[30–32] AF4 has been used as a diagnostic tool for lipoproteins and exosomes, a method for quantifying protein and DNA interactions [33, 34], and as a tool to quantify protein aggregation in therapeutic products.[35–37] The advantages and limitations of AF4 have been increasingly scrutinized its use has grown in a wide range of research disciplines and in industrial settings (as a product development tool and for quality assurance purposes). In chapter 2 of this thesis, advances in FFF characterization are reviewed with a special focus on biological, natural, and synthetic polymers. Although major strides in FFF development have been made over the past two decades several key limitations still exist. This thesis addresses these challenges which are described in the next paragraphs.

Equation 1.12 describes the normal and steric mode elution of analytes. In normal mode, small particles elute first while large particles elute first in steric mode. However, there is a size where the elution mode changes from normal to steric mode and visa versa. This transition is called the steric inversion point. Typically, the steric transition occurs at \(\sim 0.5 \, \mu m\), but can be adjusted from \(\sim 0.3\) to \(3 \, \mu m\) depending on the separation conditions used.[38, 39] This poses a problem for polydisperse samples that range from \(<0.3 \, \mu m\) to \(>3 \, \mu m\) because coelution of small particles and large particles eluting in either normal or steric mode. Chapter 3 describes the development of a simpler asymmetrical field-flow fractionation (AF4) to separate and analyze submicron (0.1 to 1 \(\mu m\)) protein aggregates and its use to gain new insights into aggregation kinetics via the Lumry-Eyring Nucleated Polymerization (LENP) model. Additionally, this work suggests AF4’s applicability over a wide size range (nanometers to tens of micrometers) could be used to analyze micrometer (\(>1\)
µm) aggregation kinetics with the appropriate detection methods. The ability to separate protein aggregates over the submicron size range provides the advances needed to identify and prevent potentially immunogenic submicron protein aggregates in biotherapeutics.

Inherent to any separation method, the sample components are subjected to dilution as one population is separated from another population. This dilution allows the dissociation constant \( K_d \) for protein and DNA binding to be determined by separation methods[40], but can also result in unwanted dissociation of weakly bound species.[41] The ability of FFF to characterize \( K_d \) into micromolar affinities highlights the techniques advantage over SEC. However, for even more weakly bound species dissociation may still occur during AF4. Often the amount of dilution during AF4 separation is thought to be the same as in SEC [21, 42], but significant differences in the separations mechanisms exist and must be taken into consideration to fully evaluate each technique. The impacts of dilution (and several other stresses) during AF4 analysis of weakly bound protein aggregates are discussed in Chapter 4. This work provides a systematic investigation of changes in analyte conditions during AF4 and invaluable insights into how these changes influence protein aggregates. An understanding of these impacts is critical for detecting and preventing aggregation in therapeutic protein formulations as well as for the characterization of other supramolecular complexes.

Perhaps the most persistent limitation of cross-flow based FFF techniques is the use of a semi-permeable membrane as the accumulation wall.[43] Typically, an ultrafiltration (UF) membrane with a nominal molecular weight cutoff (MWCO) of 1-100 kDa is used for the separation of proteins and other biological samples. However, samples with smaller sizes than the nominal MWCO can pass through the membrane lowering sample recoveries. Sample adsorption to the membrane surface presents an even greater challenge.[44] During method development, the correct membrane type and carrier fluid must be selected to limit adsorption of the analyte while still maintaining separation performance. The commercially available membrane compositions are limited and the selection of appropriate membrane/-
carrier fluid compositions may not be possible. There is a significant need for improved membranes to reduce sample adsorption and improve separation performance and reproducibility in FFF. In chapter 5, a novel reactor is designed to modify large membranes for use in AF4 channels. This work provides the critical groundwork needed to overcome the current limitations associated with analyte-membrane interactions.

The availability of sufficiently large amounts of purified analytes is critical for conducting studies concerning their fundamental properties and function. Precipitation and centrifugation have been used to isolate nanoparticles in quantities suitable for additional testing. However, precipitation may cause irreversible aggregation and sample collection after centrifugation is tedious. The development of new methods is needed to overcome these limitations. Typical AF4 channels are able to analyze \( \sim 100 \, \mu g \) sample quantities and full capabilities of new semi-preparative AF4 (sP-AF4) channels that can accommodate analyte quantities \( >100 \, \mu g \) are not fully understood. Understanding the impact of channel design and dimensions on performance of these new sP-AF4 channels and the limits of increasing sample loading is important for demonstrating their applicability. In Chapter 6, the design and performance of sP-AF4 channels are investigated and sample quantities 100 times larger than the commonly used analytical scale channels are achieved. This study of the channel design and performance provides the necessary understanding to allow for the expansion of sP-AF4 separations. Finally, chapter 7 summarizes the main conclusions and suggests future directions for further development of AF4 and other FFF technique’s.
CHAPTER 2
FIELD-FLOW FRACTIONATION FOR BIOLOGICAL, NATURAL, AND SYNTHETIC POLYMERS: RECENT ADVANCES AND TRENDS

Modified from a paper published in LCGC Europe, LCGC Asia, and LCGC North America.¹

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2.1 Abstract

Field-flow fractionation (FFF) is a family of techniques that is increasingly used for separating and characterizing macromolecules. This review discusses recent advances in the characterization of biological, natural, and synthetic polymers. Applications of FFF are contrasted with size-exclusion chromatography to illustrate practical considerations when characterizing macromolecules. The use of different FFF fields allows separations based on size, mass, composition, and architecture. The open channel design and subsequent low shear rate is well suited for analyzing weakly bound complexes, highly branched polymers, high molar mass analytes, and aggregates. Other benefits of FFF that are highlighted in this paper include simplified sample preparation, flexibility in carrier fluid choice, and on-line removal of low-molecular-weight contaminants.

2.2 Introduction

Macromolecules are ubiquitous in many areas of science and technology. Depending on the macromolecule, it is important to analyze properties like size, molar mass (M), chemical composition, degree of branching, and their respective distributions in order to understand

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Figure 2.1: Types of FFF separation (a) In AF4 a cross-flow passes through a semi-permeable membrane and porous frit. (b) In HF5 a cylindrical semipermeable membrane is used and a radial outward flow creates the perpendicular field. (c) In ThFFF a temperature gradient ($\Delta T$) is formed between a hot wall and a cold wall, and sample migrates towards the cold wall due to thermal diffusion ($D_T$).
their behavior. However, due to the complex nature of polymers, current separation techniques are not always capable of comprehensive analyses. Size exclusion chromatography (SEC) is widely regarded as the workhorse for polymer characterization, but is limited by high molar mass (HMM) macromolecules, weakly bound complexes and aggregate species, and highly branched polymers. Field-flow fractionation (FFF) is a versatile family of techniques that complements SEC with additional separation capabilities based on analyte size, mass, composition, or architecture depending on the field used (Figure 2.1). The open channel FFF design results in a soft separation mechanism that is well suited for analysis of high and ultrahigh $M$ polymers and microgel containing samples. Some key advantages of FFF over SEC are the ability to separate analytes over a broad size range (0.001 to 100 $\mu$m) using a single channel, and the absence of column packing, which greatly reduces unwanted and shear degradation. SEC of protein aggregates often requires addition of cosolvents or preconditioning of columns to reduce adsorption.[45] However, addition of cosolvents may induce aggregation, dissociate aggregates, or cause sample specific adsorption (Figure 2.2).[46] Preconditioning columns is often practiced, but not reported in literature and even when preconditioning is used poor recoveries and sample specific adsorption has been observed.[36] In FFF the ability to use formulation buffer allows separations and measurements under solution conditions that are more representative of actual use. For polymer analysis, the shear degradation and co-elution of small and large analytes observed in SEC for highly branched polymers are attributed to effects caused by the column packing material.[47]

In practice, FFF offers users additional benefits. Prior to SEC, filtering is often implemented as a sample preparation step to remove large components and help prolong the life of the column. Sample filtering has been shown to remove soluble and insoluble microgels leading to erroneous MM and polydispersity results (Figure 2.3).[48] Filtering is not required in FFF and soluble polymers and microgels can be simultaneously characterized. Many syntheses require the addition of excess reagents, which may interfere with subsequent product analyses. Such reagents or interfering low MM sample components either elute in
Figure 2.2: An IgG1 recombinant fully humanized monoclonal antibody was analyzed by FFF in two different carrier fluids (a) 0.1% acetic acid containing 50 mM magnesium chloride and (b) 10 mM phosphate buffer pH 7.1. High molar mass aggregates (peak at 18.5 min) present in (a) are absent in (b) due to weak aggregate interactions stabilized by the magnesium chloride. Reprinted with permission from (2).

Figure 2.3: ThFFF-MALS-dRI analysis of unfiltered (solid line, black symbols) and 0.5 µm filtered (dashed line, grey symbols) microgel-containing poly(vinyl acetate). The lines and symbols represent the dRI fractograms and $r_g$, respectively. Significant polymer loss in the filtered sample is evident in the lower MM distribution. Reprinted with permission from [48].
the void peak or can be removed online through a semi-permeable membrane used in some FFF techniques. Separations in FFF are dependent on the strength of an externally applied field that is easily adjusted. Therefore, resolution and separation speed are easily controlled without the need to change channels. Additionally, the open channel design greatly reduces the chance of contamination and inexpensive membranes are easily replaced when contaminated. Finally, FFF is easily coupled online with detectors frequently used for SEC analysis, including multiangle light scattering (MALS), differential refractive index (dRI), and mass spectrometer (MS) detectors. For those interested in FFF, building a simple homemade system requires an FFF channel and standard HPLC components common to many laboratories. The recent advances in FFF over the last 3 years are highlighted in this review.

2.3 Principles of FFF

Separation takes place in a thin, open, ribbon-like channel where carrier fluid transports components down the separation axis of the channel. Frictional drag at the channel walls creates a parabolic flow profile across the channel thickness, \( w \), with the fastest flows in the middle of the channel and the slowest flows near the walls (Figure 2.1). An external field (flow, thermal, sedimentation, etc.) is applied perpendicular to the separation axis of the channel to drive components towards the accumulation wall. This field-induced transport is counteracted by diffusion of components away from the high concentration region near the accumulation wall. Equilibrium is reached when the two transport processes are balanced and there is no net flux of sample in either direction. The equilibrium position is different for each sample component depending on the magnitude of their interaction with the applied field and their diffusion coefficient. Components of smaller sizes diffuse farther into the channel than larger components based on the inverse relationship between diffusion coefficient \( D \) and hydrodynamic diameter \( d_h \) given by the Stokes-Einstein equation for spherical analytes. The smaller components experience the faster flows farther from the accumulation wall and therefore elute before large components in normal mode FFF. This normal mode elution order is reverse to that observed for SEC.
The main FFF techniques relevant to this review are asymmetrical flow field-flow fractionation (AF4), hollow fiber flow field-flow fractionation (HF5), and thermal field-flow fractionation (ThFFF). AF4 utilizes a single permeable wall that allows a cross-flow to act as the perpendicular field (Figure 2.1(a)). The permeable wall is composed of a porous frit covered with a semipermeable membrane, the latter acting as the sample accumulation wall. The retention time \( t_r \) for AF4 is given by equation 2.1

\[
t_r = \frac{w^2 \pi \eta t^o \dot{V}_c d_h}{2 V^o k T} \tag{2.1}
\]

where \( \eta \) is the carrier fluid viscosity, \( t^o \) is the void time, \( \dot{V}_c \) is the cross-flow rate, \( V^o \) is the void volume, \( k \) is Boltzmann’s constant, and \( T \) is the temperature. In HF5 a semipermeable hollow fiber membrane is used and an outward radial flow acts as the perpendicular force (Figure 2.1(b)). The benefits of HF5 over AF4 are lower sample volumes and a potentially disposable channel. Thermal FFF employs a hot and cold wall to create a thermal gradient (\( \Delta T \)) and subsequently induce thermal diffusion of components towards the cold wall (in most cases) (Figure 2.1(c)). The retention time is given by equation 2.2

\[
t_r = \frac{D_T \Delta T t^o}{6 D} = \frac{D_T \Delta T \pi \eta t^o}{2 k T} \tag{2.2}
\]

where \( D_T \) is the thermal diffusion coefficient.

2.4 Biopolymers

Biopolymers are a diverse class of macromolecules that includes polypeptides, polynucleotides, and polysaccharides. The versatility of AF4 separation and characterization of biopolymers is well established. Several review papers and a book focused on biological polymers have recently been published.[31, 49, 50]

Characterizing protein-protein and protein-macromolecule complexes is important for understanding the efficacy and functions of proteins. AF4’s gentle separation mechanism is well suited to analyze complexes with weak interactions (Figure 2.4).[31] Current techniques for characterizing protein dissociation constants (\( K_d \)) such as surface plasmon resonance
(SPR), analytical ultracentrifugation (AUC), and SEC, are limited in the ability to analyze more than two components or to detect weak binding affinities ($K_d > \mu M$). Protein-protein binding between a neonatal Fc receptor (FcRn), immunoglobulin (IgG), and human serum albumin (HSA) was recently studied by AF4.[28] FcRn is involved in removing IgG proteins from lysosomal degradation pathways and IgG transportation in the body. AF4 separation of the IgG-FcRn complex allowed for the determination of a relatively low binding affinity ($K_d$ of 3.74 µM). Additionally, FcRn, HSA, IgG, and their associated complexes were separated using AF4. Using an internal standard curve the formation of multi-protein complexes were determined, including a previously unreported protein complex (HSA/FcRn/IgG/FcRn, 303 kDa). The separation of intact, weakly bound protein complexes shows great promise for AF4 studies of protein pharmacokinetics and aggregation kinetics. Analysis of protein aggregates, especially those in the submicron size range, is of particular interest in the development of therapeutic proteins. Development of an AF4 method for separating IgG monomer and submicron IgG aggregates was recently shown by Hawe et al.[36] Better size resolution and recoveries of submicron IgG aggregates were achieved by AF4 compared to SEC.

Figure 2.4: Comparison of FFF to other currently used techniques (analytical ultracentrifugation - AUC; surface plasmon resonance - SPR) for protein-protein characterization. The open channel FFF design and flow-based separation extends the current ability to detect weak protein-protein interactions into the µM binding affinity range. Reprinted with permission from [28].
Lipoproteins are assemblies of proteins and lipids that function as carriers for lipids and cholesterols in blood. AF4 has been used to analyze low-density lipoproteins (LDL) and high-density lipoproteins (HDL).[51] LDL has been associated with increased risk of coronary artery disease (CAD). In addition to the conventional AF4 channel, a hollow fiber guard channel placed before the AF4 channel was evaluated using serum from healthy patients and CAD patients. The guard channel removed contaminants and improved reproducibility in retention, and fluorescence detection reduced adsorption of serum proteins to the membrane and reduced the amount of serum required per injection (0.13 µL).

Online coupling with a variety of detection methods has expanded the breadth of AF4’s characterization ability in recent years. The use of MALS, dRI, and quasi-elastic light scattering (QELS) detectors has become more common for characterizing macromolecules. Characterization of dh, MM, radius of gyration ($r_g$), and chemical composition was shown for a PEGylated protein conjugate and its aggregates using online AF4-UV-MALS-QELS-dRI, SEC-UV-MALS-QELS-dRI, and matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) as complementary techniques.[52] PEGylated protein, unreacted protein traces, and aggregated species were detected by both AF4 and SEC, with AF4 providing superior size resolution, while MALDI-TOF-MS was unable to detect aggregates. The UV-MALS-QELS-RI detection enables chemical composition characterization of PEGylated proteins (1/1 PEG to protein ratio) and allowed identification of aggregates present using different storage buffers.

Interest in characterizing protein complexes by 2D offline coupling of AF4 with other separation techniques and a variety of detection methods has grown in recent years. Lectin treated N-linked glycopeptides in serum from lung cancer and healthy patients were separated by AF4 and subsequently analyzed by nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS-MS).[53] Binding of various lectin types to glycoproteins enables a size-based separation by AF4. Removal of non-lectin bound glycopeptides and size sorting of lectin-glycopeptide complexes during AF4 allowed semi-quantitative anal-
ysis and improved identification of biomarkers by nLC-ESI-MS-MS. Similarly, characterizing cholesterols and triglycerides in lipoprotein complexes is also important for understanding their function in the body. Offline coupling AF4 and gas chromatography (GC)/MS allowed cholesterols and triglycerides to be profiled from human serum samples, and results showed agreement with the current enzymatic determination methods.[54]

The use of FFF has shown promise as a pre-MS separation technique in proteomics analyses. To improve MS detection of poorly soluble proteins, the effect of protein-SDS complexation on protein solubility was examined by HF5 and nLC-MS.[55] SDS-denatured serum samples, unfractionated or fractionated, showed improved solubility of the SDS-protein complexes, and allowed for a greater number of proteins to be identified by nLC-MS. Furthermore, the HF5 process was shown to remove low $M(<30\text{ kDa})$ components, which subsequently led to lowered background noise in the MS spectrum.

Protein phosphorylation is a post-translational modification, which plays an important role in protein regulation and can be used as biomarkers for diseases like cancer. The 2D online coupling of an isoelectric focusing (IEF) step and AF4 step prior to nLC-ESI-MS-MS enabled separation of phosphorylated proteins from a proteome sample based on isoelectric point (pI) and $d_h$.[57] IEF-AF4 separation was evaluated for unphosphorylated and phosphorylated -casein. Peptides with higher degrees of phosphorylation eluted in the lower pH channels and at longer AF4 retention times as expected. Relative abundances of phosphorylated protein biomarkers were determined by IEF-AF4 and nLC-ESI-MS-MS for a prostatic cancer line and a normal cell line. In another study, improvements in direct online coupling of AF4 with ESI-MS were shown in a small chip-type channel for top-down proteomics that operates in the micro flow rate regime (Figure 2.5).[56] The chip-type channel effectively separated carbonic anhydrase (29 kDa) and transferrin (78 kDa) while using much lower, and more ESI-MS compatible, channel flow rates (<12 $\mu$L/min) than previous online studies (Figure 2.5(b)).[32, 58] Resolution of monomer and aggregate species as well as desalting during AF4 led to higher signal to noise for ESI-MS detection (Figure 2.5c and
Figure 2.5: A schematic of the chip-type miniaturized AF4 channel interfaced with electrospray ionization mass spectrometry (AF4-ESI-MS) (b) Base peak fractogram (BPF) of AF4-ESI-MS for the separation of CA and transferrin ($\dot{V}_{out}/\dot{V}_c = 0.012/0.49$ mL/min) (c) Full scan ESI-MS for peak number 2 (transferrin) after AF4 shown in Figure 5b (d) Full scan ESI-MS spectrum of transferrin (0.01 µg/µL) without AF4. Considerably better S/N is observed in the fractionated transferrin due to monomer/dimer resolution and contaminant removal during AF4. Reprinted with permission from [56].
Lipodomic analysis of HDL and LDL from human serum was also shown by chip-type AF4.[59] Online desalting improved ionization of the HDL/LDL lipids, and in a CAD plasma sample twenty-eight phospholipids, eighteen triacylglycerides, and six cholesteryl esters were identified.

### 2.5 Natural Polymers

Starches are macromolecules essential to human beings and used in a variety of industrial and food applications. The properties of starches (digestion, thickening properties, etc.) are dependent on starch structure, which in turn is dependent on the degree of branching. However, wide size distributions and variation in branching makes characterizing starches difficult.[60] AF4 coupled with MALS and dRI detectors have been successfully used to determine starch $d_h$, $M$, and $r_g$.[61, 62] An in depth review of FFF characterizing food macromolecules has been recently published.[63]

Wahlund et al. demonstrated the power of AF4-MALS-RI to rapidly separate amylose and amylopectin in maize, wheat, rice, potato, and tapioca starches.[64] Qualitative results for amylose/amylopectin ratios demonstrate the feasibility for relatively fast characterization of starches by AF4 and provide a starting point for more extensive starch analyses. Studies by Juna et al. have examined various starches (waxy maize, tapioca, corn, sago) to better understand AF4 conditions and starch processing parameters.[65–70] Changes in size distributions were observed with changes in AF4 conditions. For example, at high cross-flow rates, tapioca, sago, and corn starch $d_h$, $M$, and $r_g$ distributions shifted to lower values due to increased retention (or potentially degradation of HMM components). The effect of AF4 conditions is important and must be considered for accurate analyses of starches.

Coupling a separation technique with MALS and dRI detectors can provide information on structural and branching characteristics of starches. SEC is the most common separation technique used to characterize starches, but low exclusion limits and shear scission may bias results. AF4 has the potential to reduce artifacts observed in SEC such as changes in $M$ and size distributions due to shear degradation or aggregation and large branched polymers.
that co-elute with smaller components.\cite{47, 60} A more in depth comparison of AF4 and SEC as separation techniques for starches is available.\cite{71} To characterize size distributions and gain structural information for a commercial starch and a waxy yam starch, Perez et al. compared AF4-MALS-dRI and SEC-MALS-dRI.\cite{72} AF4 and SEC results both yielded smaller sizes for the commercial starch than the waxy yam starch, while a more quantitative recovery for AF4 (100\%) was seen compared to SEC (62\%). Structural characterization of the starches was also accomplished by SEC and AF4. Plotting the $r_g$ and $M$ of the same fraction, and using the exponent, $\nu_g$, from the equation $r_{gi} = K_g M_i^{nu_g}$ where $K_g$ is a constant, the polymer shape can be described ($\nu_g$ of 0.3, 0.5-0.6, and 1, describe the polymer shape for a sphere, a linear random coil, and a rod, respectively). Values for AF4 and SEC were all close to 0.4, which fell between a sphere and a random coil. Rolland-Sabat et al. examined the differences between hydrodynamic chromatography (HDC)-SEC and AF4 for characterizing starches.\cite{73} Better separation of amylose and amylopectin was achieved with AF4 and allowed determination of $d_h$ and $M$ distributions and better structural characterization (especially for large amylopectin fractions). Additionally, the branching parameter distributions showed that WTPS and WTRS amylopectins could be discerned by AF4, but not HDC-SEC.

Characterizing aggregates is important for understanding the solution behavior and physical properties of polysaccharides. Arabinoxylan and its aggregates were characterized by AF4-MALS-dRI and SEC-MALS-dRI.\cite{74} Although aggregate concentrations were low, co-elution of individual polymers and aggregates in SEC led to larger molar masses and $r_g$s reported compared to AF4. Differences in glucan linkages may also change its properties to allow for new uses as a bio-based polymer. The $M$, size, and conformation of dextrans with varying amounts of $\alpha(1-3)$ glycosidic linkages has also been investigated.\cite{75} Using $\nu_g$ values, dextrans containing the most $\alpha(1-3)$ linkages were found to be the smallest and densest while dextrans with the least amount $\alpha(1-3)$ linkages displayed a quasi-linear conformation. Understanding the physical and structural properties of glucan allows for further development.
of biomaterials.

Figure 2.6: AF4 fractograms of barley-å-glucan (lines represent fluorescence and symbols represent molar mass). Samples dispersed in water (grey-dashed line, circles), after in vitro gastric digestion (gray full line, squares), and undergoing additional small intestinal digestion (black line, triangles) were analyzed by AF4-MALS. Gastric digestion samples show a reduction in aggregate species, while the re-formation of higher density is shown after small intestinal digestion. Reprinted with permission from [76].

β-glucans solution behavior and ability to form aggregates may be associated with beneficial health effects. For example, understanding β-glucan digestion aids in the understanding the physiological effects of soluble fiber. Several recent studies have used AF4-MALS-dRI to analyze β-glucans.[76–78] In one study, β-glucan aggregates under gastric digestion conditions were disrupted while, after undergoing small intestinal digestion, aggregates were reformed (Figure 2.6).[76] The disruption and re-formation of aggregates is likely to impact the behavior and function of β-glucan. To demonstrate the effect of processing and storage on aggregates, Ulmius et al. subjected barley β-glucan samples to several conditions (storage time, heating, freeze time, freeze-thaw, solution) and performed AF4-MALS-dRI analysis.[77] Disruption, structural change, or elimination of β-glucan aggregates was observed under most conditions. Properties of individual and aggregated β-glucans from oat and barley were also compared using AF4-MALS-dRI.[78] Individual molecules could be
distinguished from supramolecular species based on conformational differences across the size distribution. Additionally, dissolution of both β-glucans under harsh alkaline conditions showed that barley β-glucan aggregates were not dissolved as previously proposed.

AF4 has been applied to hyaluronan (HA) and sodium hyaluronate (NaHA) polysaccharides that have important biological functions and industrial applications.[79] Characterization of HA $M$ and conformation by AF4-MALS-dRI yielded results that were consistent with other methods, including SEC-MALS-dRI. Both AF4 and SEC were able to measure low $M$ ($<1\times10^6$ g/mol) samples. Molar mass distributions, an important parameter for HA characterization, were also similar between AF4 and SEC measurements. NaHA is used commercially in pharmaceutical and cosmetic products.[80] Molar mass distributions and structural properties of NaHA and commercially blended NaHA mixture were characterized and compared by frit inlet (FI) AF4-MALS-dRI. Frit inlet is a particularly gentle FFF method without the initial focusing step. Significant aggregation was not observed while samples subjected to gamma ray sterilization showed a significant breakdown of NaHA.

Exudate gums are complex polysaccharides with industrial applications. They are used as emulsifiers and stabilizers and contain a small amount of proteinaceous material. Molar mass, $r_g$, $d_h$, conformation, apparent densities, and distribution of proteinaceous material were determined for gum arabic (GA) and mesquite gum (MG) by AF4-MALS-dRI.[81] The separation of polysaccharide and proteinaceous populations, and the characterization of important molecular data over the entire size range were demonstrated by AF4. Using AF4, it was possible to conclude that GA-stabilized emulsions were more stable against coalescence than MG-stabilized emulsions.

The characterization of gelatin by AF4 has also been shown.[82] In denatured native gelatin an increase in $M$ during renaturation was attributed to α, β, and γ chain interactions. However, an increase in $M$ for thermally pre-treated gelatin was not seen indicating an inhibition of α, β, and γ chains in gelatin limiting renaturation. The effect of available lysine (lysine with a hydrogen bonding amino group) on formation of $HMM$ compounds

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in gelatin was also characterized by AF4.[83] A decrease in available lysine with thermal treatment led to higher molar masses.

Tannins play an important role in the color, taste, and overall quality of wine. Oxidized tannins formed macromolecules and were characterized by AF4-MALS showing soluble and insoluble populations.[84] Both AF4 and small-angle X-ray scattering (SAXS) showed that the $M$ of insoluble macromolecules was much higher than the soluble macromolecules.

### 2.6 Synthetic Polymers

In the last several years, advances in the characterization of synthetic polymers have included the introduction of an elevated temperature AF4 instrument and new applications in AF4 and thermal FFF. Low $M$ polyethylene samples and a number of narrowly distributed polystyrene standards were analyzed by AF4-MALS-dRI in organic solvent and compared to SEC-MALS-dRI.[24] At ambient temperature, low density polyethylene, polypropylene and polybutadiene, containing high degrees of branching and $HMM$, have been analyzed. Due to co-elution of large and small macromolecules in SEC, a correct calculation of the $M$ distribution and the $M$ average or branching ratio is not possible (Figure 2.7). In contrast, AF4 allows the precise determination of the $M$ distribution, the $M$ averages and the degree of branching because the $M$ vs. elution volume curve and the conformation plot is not affected by the co-elution issues encountered in the SEC analysis.

In addition, due to the absence of significant shear degradation in the channel, characterization of linear and branched $HMM$ polyethylene by AF4 has been developed under high temperature (HT) conditions (145°C) in organic solvent (1,2,4-trichlorobenzene (TCB).[25] Compared to HT-SEC, HT-AF4 allows for a more complete separation of highly branched polyethylene with limited co-elution of large and small macromolecules. The HT-AF4 technique coupled with MALS detection was used for quantification and size determination of the co-eluting molecules. Furthermore, HT-AF4 induced lower shear and thermo-oxidative degradation of $HMM$ PE and PP than HT-SEC.[26] As a consequence, the $HMM$ averages obtained from HT-AF4 are significantly higher than those obtained from HT-SEC. It was
Figure 2.7: Separation of a low density polyethylene sample (CSTR-LDPE 1) by HT-AF4 and HT-SEC, with MALS and dRI detection. The abnormal curvature of the molar mass from HT-SEC indicates co-elution due to the high branching of the polymers. AF4 shows complete separation over the entire size range into the ultrahigh molar mass range not detected by SEC due to shear degradation. Reprinted with permission from [24].

shown that most of the observed limitations of SEC could be overcome by using AF4.

AF4 has also been used for dendritic polymer characterization. Different poly(amideamine) (PAMAM) dendrimers have been characterized by AF4-MALS,[85] The separation between different generations (4 to 9) of PAMAM particles has been shown under different pH conditions and AF4 highlighted the presence of some impurities. Coupled with other on-line characterization techniques (eg., MALS or a differential viscometer), AF4 allows for more detailed physical characterization of each separated size fraction. Aggregation and complexation of dendritic glycopolymers, used as drug delivery systems, has been demonstrated using AF4-MALS.[86–88] Additionally, removal of small sample components through the ultrafiltration membrane during AF4 can be used to quantitatively determine the amount of complexed small guest dye molecules in core-shell polymers. This feature of AF4 can potentially be used for the separation and quantification of drugs encapsulated in polymers and makes the AF4 technique very promising for the analysis of drug delivery systems.

Other kinds of drug delivery systems such as micelles have been characterized by AF4. Poly(ethyleneoxide-β-ε-caprolactone) (PEO-β-PCL) self-assemblies in water were character-
ized by AF4 with on-line MALS-dRI-UV/Vis-QELS detection.[89] This study underlined the impact of the mass of the PEO and PCL fragments on the micelle size. Hydrodynamic radii measured by QELS were in good agreement with values calculated by AF4 retention times. AF4 illustrated that in some cases the number of self-assemblies present was very low compared to the number of unassembled diblock copolymers. Finally, quantification of photosensitizers used in photodynamic therapy encapsulated by these micelles has been done. This approach was used to characterize several diblock copolymer micelles (PEG-PVP, PEG-PLA, PEG-PLGA and PEG-PCL) and determine their in vitro half-lives in human serum.[90] The impact of human serum on the micelle size and stability was shown by AF4. Indeed, micelle disassembly was observed for PEG-PVP micelles whereas PEG-PLA, PEG-PLGA and PEG-PCL micelles were far more stable.

Thermal FFF has been mainly used to fractionate and characterize lipophilic polymers in organic solvents. The applied force is a temperature gradient that causes thermal diffusion of analytes. The magnitude of the thermal diffusion coefficient $D_T$ has been empirically observed to depend on the polymer-solvent interface and other factors.[91–93] Thermal diffusion in liquids is a complex phenomenon that is not yet fully understood.[94–96] However, its usefulness in ThFFF polymer separations has been demonstrated and new interesting capabilities are being developed. For example, the observation that different polymer chemistries in the same solvent or the same polymer chemistry in different solvents can have different $D_T$ and hence $t_r$ (see Equation 2.2) allows for chemical composition (in addition to size) analyses of polymers.

ThFFF coupled with MALS-dRI-QELS was used to simultaneously determine the MM and composition of polystyrene-poly(n-butyl acrylate) (PSPBA) and polystyrene-poly(methyl acrylate) (PSPMA) copolymers (Figure 2.8(a) and (b)).[94] Equation 2.2 shows that the retention time is proportional to $D_T/D$. If $D$ can be measured independently, i.e., by QELS, $D_T$ can be calculated. When on-line $D$ measurements are made, $D_T$ can be calculated as a function of $t_r$ and subsequently correlated with polymer composition. Using this premise,
the $D_T$ was found to be independent of $M$ for copolymers with similar compositions and dependent on composition of copolymers with similar $M$ in a non-selective solvent. The ThFFF-MALS-dRI-QELS combination allowed rapid determination of copolymer $M$ and chemical composition distributions. ThFFF has recently been coupled to NMR off-line [97] and on-line [98] in the analysis of triblock copolymers and PS, poly(methyl methacrylate) (PMMA), polyisoprene (PI), and PS-$b$-PMMA block copolymers, respectively. NMR provided an independent measurement of copolymer composition and confirmed compositional separation by ThFFF.

![Graphs](image)

Figure 2.8: Weight percent composition of (a) PS-PBA and (b) PS-PMA copolymers were determined through averaged online DT measurements. ThFFF weight percent values are consistent with the nominal weight percent values. Reprinted with permission from [94].

To date, ThFFF method development has been predominantly through trial-and-error based on other published work. A recent paper demonstrated that a theoretical approach based on temperature dependent osmotic pressure gradient and polymersolvent interaction parameters can be used to successfully estimate $D_T$ and retention times for different polymersolvent pairs.[93] Experiments confirmed the calculation of poly(n-butyl acrylate) (PBA), poly(methyl acrylate) (PMA) and PS retention times in different solvents. This provides a potential route to predicting good solvents for polymer retention.
Thermal diffusion is an intriguing phenomenon with hidden potential for other important analyses. In a recent development, it has been shown that the correlation between theoretical and experimental $D_T$ values can provide information about the number of chain ends for branched polymers. Runyon thesis The uniqueness of this study lies in that the chain ends could be determined without the need for a linear polymer analog. The ThFFF-MALS-dRI-QELS combination allowed simultaneous determination of $M$, composition, and number of chain ends.

2.7 Conclusions

FFF is a versatile family of techniques for characterizing biological, natural and synthetic macromolecules. As a complementary technique to SEC, more detailed macromolecule characterizations are possible using both FFF and SEC. FFF’s open channel design and soft separation mechanism make it a powerful technique for analyzing weak macromolecule interactions, polymer aggregates, and $HMM$ and highly branched polymers. The benefits to users are also evident in simplified sample preparations, ultrafiltration of contaminants during separation, and flexibility in carrier fluid choice among others. With the 16th International Symposium on Field- and Flow- Based Separations in Pau, France this July, more interesting developments are anticipated along with a flurry of associated publications.

2.8 Acknowledgments

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CHAPTER 3
PROBING SUBMICRON AGGREGATION KINETICS OF AN IgG PROTEIN BY
ASYMMETRIC FLOW FIELD-FLOW FRACTIONATION

Modified from a paper published in *Journal of Pharmaceutical Sciences*.\(^4\)

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3.1 Abstract

A lack of reliable analytical methods has hindered the quantification of submicron protein aggregates and a detailed understanding of their formation kinetics. In this study, a simple asymmetric flow field-flow fractionation (AF4) method with good size selectivity (>0.5) is used to investigate nanometer (<0.1 \(\mu\)m) and submicron (0.1 to 1 \(\mu\)m) aggregates of heat stressed anti-streptavidin (anti-SA) IgG. The Lumry-Eyring Nucleated Polymerization (LENP) model for nonnative protein aggregation is fit to the AF4 data and kinetic analysis show that aggregates are formed via slow nucleation and aggregate condensation at long stress times. Comparison of centrifuged and uncentrifuged heat stressed anti-SA IgG1 AF4 results show the removal of high molar mass submicron aggregates and large material (>20 \(\mu\)m) and suggests that centrifugation may influence the aggregation kinetics. Furthermore, qualitative LENP model analysis of centrifuged and uncentrifuged samples suggests that significant aggregate-aggregate condensation occurs even at early stress times and highlights the potential of AF4 to determine aggregation kinetics for species >1 \(\mu\)m.

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3.2 Introduction

In the past 20 years, the use of therapeutic proteins has grown rapidly for the treatment of diseases including cancer, Crohn’s disease, and rheumatoid arthritis, among others.[99] During production, transportation, and storage, therapeutic proteins experience a variety of stress conditions that may result in the formation of protein aggregates.[100] Aggregation of therapeutic proteins is a major concern because of the reduced product efficacy and potential immunogenicity.[1] However, developing stable therapeutic protein formulations and understanding aggregate formation is a challenge due to the complex nature of proteins.

Protein aggregates can range from small nanometer (<0.1 µm) to larger submicron (0.1 to 1 µm), micrometer (1 to 100 µm) and visible (>100 µm) particles making their characterization difficult by a single analytical technique.[14, 101–105] The United States Pharmacopeia (USP) <788> specifies methods for counting subvisible particles >10 µm in therapeutic formulations.[106] Visual inspection is commonly used to detect particles >100 µm and light obscuration (LO) and optical microscopy are described in the USP for quantifying particulates >10 µm. In recent years, flow imaging techniques such as Microflow ImagingTM (MFI) and FlowCamTM have become more common for quantifying particles from 1 to 10 µm.[15–17, 104, 107] Using a CCD camera, images are captured as the sample flows through the field of view of a microscope. Image analysis is then used to determine particle size, number, shape, and transparency. One significant advantage in flow imaging analysis is the discrimination between proteinaceous and non-proteinaceous particles such as silicone oil droplets.[108] Enabling technologies such as new ethylene tetrafluoroethylene polymer particle standards with a similar refractive index (1.40) as proteins have been developed to allow more accurate size analysis.[109, 110] Despite these recent advances, the lower size limit of flow imaging techniques remains at 1 µm.

The submicron (0.1 to 1 µm) aggregate size range is an area of particular interest due to the lack of reliable analytical techniques and potential immunogenicity of aggregates.[2, 100, 111] Dynamic light scattering (DLS) is commonly used for submicron analysis and relies on
the correlation between fluctuations in light scattering intensity and the analytes diffusion coefficients \((D)\) and, in turn, their hydrodynamic diameters \((d_h)\). DLS results are often biased towards larger sizes due to the higher scattering intensity \((I \propto d^6)\) of large particles and contaminants like dust.[11, 104] Furthermore, DLS is a low resolution technique and particle populations must differ in size by a factor of at least three to be resolved.[11] Nanoparticle tracking analysis, (NTA) utilizes a CCD camera and laser illumination to capture the Brownian motion and light scattered by individual particles. Subsequent software analysis yields \(D\) and \(d_h\) values. By tracking individual particles, NTA is able to distinguish particles of similar sizes better than DLS and provides semi-quantitative particles counts.[11] NTA analysis has an optimum aggregate concentration range between \(10^7\) and \(10^9\) particles/mL [11, 112] and a lower size limit of 40 nm (for low refractive index particles such as protein aggregates). The upper size limit is often reported as 1 \(\mu\)m, although in practice, particles above 0.5 \(\mu\)m are often removed by filtration or centrifugation to minimize scattering interference that can mask the signal from smaller particles. Resonance mass measurement (RMM) is another recently introduced method for quantifying protein aggregates.[13] It is performed by flowing analytes through a microchannel resonator that is suspended in a vacuum chamber. Changes in the resonant frequency as particles pass through the channel are correlated with their buoyant mass. Silicone oil droplets and protein particles can be distinguished by RMM, but submicron aggregate analysis is hindered by a lower size limit of 0.4 \(\mu\)m, potential clogging of the microfluidic channel by large particles, and the need to assume protein aggregate density. Flow cytometry and Coulter counter have also been used to analyze the submicron aggregates region.[113, 114] However, both techniques are limited to particles >0.5 \(\mu\)m and high conductivity electrolyte solutions required for Coulter counter analysis may alter aggregate populations thereby limiting its utility for aggregate quantification in formulation buffer.[14, 100, 102, 104]

The complexity (size, shape, and density) of protein aggregates necessitates the incorporation a separation stage into the analysis of these complex samples. Size exclusion chromatog-
raphy (SEC) is the most widely used technique to separate and quantify protein monomer and nanometer (<0.1 µm) aggregates.[14] However, well known limitations exist for the SEC analysis of protein aggregation.[115] Interaction of analytes with the column packing can lead to changes in elution times, peak shapes, and sample recoveries. Adjusting the mobile phase ionic strength and using additives, such as arginine or organic modifiers, can reduce interactions, but may also affect the aggregate subpopulations present in the original formulation.[45] Protein formulations often include excipients, stabilizers, and additives and loading these samples onto the SEC column can influence column stability and reduce the column lifetime.[19] The inherent shear forces experienced during SEC analysis especially under high pressures (>100 bar) can also lead to changes in aggregate populations especially for large and weakly bound species. In one SEC study, increased amounts of aggregates were generated when using separation conditions of 410 bar compared to 125 bar.[20] In addition to sample changes caused by SEC columns, aggregates are often removed by pre-column frits or centrifugation during sample preparation to avoid plugging the column.[22] These sample preparation steps can eliminate submicron sample components of interest. Analytical ultracentrifugation is a complementary technique to SEC for nanometer size aggregates, but is limited by low throughput and complicated data analysis.[14]

The limitations of SEC and other techniques outlined above can be addressed by asymmetric flow field-flow fractionation (AF4).[10, 36] The open AF4 channel (no packing material) translates to reduced undesirable interactions and sample loss, low pressures (<15 bar) and low shear rates that allow weakly bound protein complexes and aggregates to be characterized. Pollastrini et al. showed that AF4 was able to quantify weak binding (K_d >1 µM) of an IgG/FcRn complex that was not measurable by SEC.[28] Additionally, there is more flexibility in the choice of carrier liquid in AF4 (aqueous and organic channels are commercially available) and the formulation buffer can often be used.[28, 37, 45] This is an important advantage as the carrier liquid composition can dramatically affect both protein structure and aggregate formation.[46]
AF4 has been used to investigate the effects of protein formulation stability and accelerated protein aggregation.[36, 46] A variety of accelerated stress conditions including pH, freeze-thaw, and heat can cause aggregate formation. Despite its many advantages, AF4 has not been used to determine submicron nonnative aggregation kinetics. Nonnative aggregation (from here on referred to simply as aggregation) involves a change in conformation from the native protein and is a major source of degradation products in processing, packaging, transport, storage, and administration of protein therapeutics.[5, 6, 101, 116, 117] Understanding aggregation kinetics is important for controlling and eliminating aggregate species to ensure product safety.[1] A number of models exist that incorporate aggregate nucleation and growth steps to describe protein aggregation kinetics.[118–124] Many of these models are included as limiting cases in a general model for protein aggregation called the Lumry-Eyring Nucleated Polymerization (LENP) (Figure 3.1).[125–129] This model, developed by Roberts and co-workers, is highly suited for this work because experimentally relevant quantities such as monomer fraction (m) and molar mass (M) are measurable by AF4.

A simplified schematic of the LENP model[130] is shown in Figure 3.1. Aggregate formation involves a series of processes beginning with conformational changes in native monomer (N) proteins to form reactive monomer (R) conformers that subsequently self-assemble to form reversible oligomers (Rx) composed of x number of monomers. These reversible oligomers rearrange to form the smallest (essentially) irreversible aggregate nucleus (Ax).

The LENP model considers the nucleation step to be irreversible if significant stabilizing interprotein interactions occur and/or if aggregate growth is much faster than nucleation. Aggregate growth can occur by two mechanisms. Chain polymerization aggregate growth occurs via addition of one or more reactive monomer species (Rδ), where δ is the number of monomers added per growth event. Growth may also occur by aggregate condensation where aggregate species (Aj), where j is some number of monomers greater than x, associate to form larger aggregates and eventually insoluble aggregates that phase separate. The description of the LENP model above is brief as existing publications discuss the model in
The LENP model consists of a system of coupled differential equations that can be solved numerically (Eqns. 3.1-3.3). Regression of these solutions against experimental data, m and M with respect to time (t), yield characteristic time constants for nucleation (τₙ), chain polymerization growth (τₙ), and aggregate condensation growth (τₑ). These time constants are inversely proportional to rate constants for each stage. The change in m with respect to t is described by Eq. 3.1

\[
\frac{dm}{dt} = -x \frac{m^x}{\tau_n} - \frac{\delta}{\tau_g} m^\delta \sigma
\]  

(3.1)

where σ is the total aggregate concentration over the initial monomer concentration. As Aₓ species form via nucleation, they undergo chain polymerization growth that consumes both R and Aₓ. Equation 3.1 indicates that nucleation and chain polymerization consume Rₜ, in
turn decreasing $m$ over time. The change in $\sigma$ with respect to $t$ is described in Eq. 3.2.

$$\frac{d\sigma}{dt} = \frac{m^x}{\tau_n} - \frac{1}{2\tau_c} \sigma^2$$  \hspace{1cm} (3.2)

Nucleation is the driving force for increasing the number of aggregate species. Eq. 3.2 illustrates that the greater the rate of nucleation, the faster $\sigma$ increases. Conversely, aggregate condensation consumes aggregate species present in solution and thus decreases $\sigma$ over time. Chain polymerization refers to the growth of pre-formed nucleation species and does not affect $\sigma$. Changes in the second moment of the aggregate size distribution ($\lambda_2$) with respect to $t$ are described in Eq. 3.3.

$$\frac{d\lambda_2}{dt} = x^2 \frac{m^x}{\tau_n} + \frac{1}{\tau_g} m^\delta (\delta^2 \sigma + 2\delta(1-m)) + \frac{1}{\tau_c} (1-m)^2$$  \hspace{1cm} (3.3)

The second moment of the aggregate size distribution is related to $m$ and $M$ by Eq. 3.4

$$\frac{M^{agg}}{M^{mon}} = \frac{\lambda_2}{1-m}$$  \hspace{1cm} (3.4)

where $M^{mon}$ is the molar mass of the monomer and $M^{agg}$ is the weight average molar mass of the aggregate species. The $M^{agg}/M^{mon}$ parameter provides an experimentally accessible quantity to describe aggregate growth in addition to $m$. From Eq. 3.3 it can be seen that nucleation, chain polymerization, and aggregate condensation contribute to increases in $\sigma$. Eqns. 3.1-3.3 use the simplifying approximation that the aggregation mechanism is size-independent and hold true under conditions when nucleation is slow compared to the rate of aggregate growth.[129]

SEC has been used most frequently to characterize the aggregation kinetics of oligomer and high $M$ species.[127, 129, 130] However, heat stressed protein samples at pHs >5.5 often produce submicron, micrometer, and visible species that are too large for SEC analysis.[36, 128, 131] To the authors knowledge, there has been only one study where both SEC and AF4 were used to characterize aggregation kinetics of small oligomers[132] and no studies demonstrating the ability of AF4 to characterize aggregation kinetics of high $M$ aggregates.

In this work, an AF4 method is developed to characterize the aggregation kinetics of a heat
stressed anti-streptavidin (anti-SA) IgG1 antibody over the nanometer and into the submicron size ranges using the LENP model. Additionally, AF4 analysis is used to qualitatively compare aggregation kinetics of anti-SA samples with and without centrifugation to provide additional insights into the aggregation mechanism.

3.3 Materials and Methods

Purified anti-streptavidin (anti-SA) IgG1 was provided by Amgen (Thousand Oaks, CA) in formulation buffer at 30 mg/mL. NaH$_2$PO$_4$ • H$_2$O and Na$_2$HPO$_4$ (anhydrous) were purchased from Fisher Scientific (Fair Lawn, NJ). NaN$_3$ was purchased from Sigma Aldrich (St. Louis, MO). Phosphate buffer (100 mM, pH 6.25) was prepared by mixing 10.99 g of NaH$_2$PO$_4$ • H$_2$O, 3.12 g of Na$_2$HPO$_4$ (anhydrous), and 0.20 g of NaN$_3$ in deionized water (APS Filtration AquaMax Ultra, Buffalo, NY). Phosphate buffer filtered through 0.22 µm mixed cellulose ester filters (Millipore, Bedford, MA) was used for anti-SA sample dilution and as the AF4 carrier fluid.

Anti-SA samples of 1 mg/mL in were prepared in 15 mL centrifugation tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and filtered using 0.45 um PVDF filters (Whatman, Florham Park, NJ). Aliquots (0.5 mL) of anti-SA were then transferred to 1.5 mL centrifuge tubes (Sorenson BioScience Inc., Salt Lake City, UT) for heat stress treatment. Aliquots were stressed at 67°C in a PolyScience 8000 Constant Temperature Circulator (Niles, IL) and immediately quenched for 5 minutes in an ice bath, stored at 2-8°C and analyzed within 24 hours. Sample solutions remained clear after ice bath quenching. Stressed and quenched samples were not filtered or centrifuged unless otherwise stated.

A lyophilized IgG protein was purchased from Sigma Aldrich (St. Louis, MO), diluted in phosphate buffer to 1 mg/mL and used for the analysis in Figure S1. The sample was not heat stressed, centrifuged, or filtered prior to analysis.
3.3.1 Asymmetric Flow Field-Flow Fractionation (AF4)

An AF2000 system (Postnova Analytics, Salt Lake City, UT) was coupled with an SPD-20A UV/Vis detector at 280 nm (Shimadzu, Kyoto, Japan), a multi-angle light scattering (MALS) DAWN EOS, and an Optilab T-REX refractive index detector (Wyatt Technologies, Santa Barbara, CA). The AF4 channel was formed using a 350 µm trapezoidal spacer with a length of 29.4 cm, maximum width of 2 cm and minimum width of 0.5 cm. A 30 kDa molecular weight cutoff regenerated cellulose membrane (Micodyn Nadir, Wiesbaden, Germany) was used as the accumulation wall and the sample injection loop was 20 µL. Sample focusing was performed for 3 minutes using an injection flow rate of 0.2 mL/min, a cross-flow rate of 1.0 mL/min, and an outlet flow rate of 0.5 mL/min. Elution conditions employed an outlet flow rate of 0.5 mL/min and a cross-flow rate that was varied from 1.0 mL/min to 0.1 mL/min over 10 minutes and then held constant at 0.1 mL/min for 15 minutes. The cross-flow was turned off at the end of each run to assess sample recovery and minimize the possibility of cross contamination.

3.3.2 MALS data treatment

The M and root mean square radii \( r_{rms} \) of sample components eluting from the AF4 channel were determined by MALS. The intensity of the scattered light measured at various angles \( \theta \) and the sample concentration \( c \) obtained by UV detection is fitted by the Berry equation [133]

\[
\sqrt{\frac{Kc}{R_\theta}} = \sqrt{\frac{1}{M} + \frac{16\pi^2}{3\lambda^2} \frac{1}{M} (r_{rms})^2 \sin^2\left(\frac{\theta}{2}\right)} \tag{3.5}
\]

where \( R_\theta \) is the excess Rayleigh ratio (proportional to the scattered light intensity at each \( \theta \)), \( M \) is the weight average molar mass, \( K \) is an optical constant, and \( \lambda \) is the incident wavelength. The Berry equation has been shown to be more accurate than the Zimm or Debye equations for analytes with sizes approaching the wavelength of the laser (690 nm) or of unknown shape.[134]
3.3.3 LENP Data Treatment

Aliquots of anti-SA were heat stressed for times ranging from 0 to 30 minutes and then analyzed by AF4. For each stress time, the m (monomer fraction), $M^{\text{agg}}$ (weight average molar mass of the aggregate peak), and $M^{\text{mon}}$ (monomer molar mass) terms in Eqns. 3.1-3.4 were determined from AF4 experiments. The $m$ is determined by taking the peak area (PA) of the monomer at a given stress time and dividing it by the PA of the monomer peak in the unstressed sample. The $M^{\text{agg}}$ and $M^{\text{mon}}$ are determined by MALS using Eq. 3.5. In the LENP, $M^{\text{agg}}/M^{\text{mon}}$ represents the number of monomers per aggregate and is related to $\lambda_2$ (the second moment of the aggregate size distribution) by Eq. 3.4. Numerical integration of Eqns. 3.1-3.3 by a fourth order Runge-Kutta method and regression to $m$ and $M^{\text{agg}}/M^{\text{mon}}$ is performed using Wing IDE 5.0.2-1 (Archaeopteryx Software, Inc, Cambridge, MA).

3.4 Results and Discussion

Optimization of the AF4 method, the effects of heat stress, the impact of sample centrifugation, and the determination of aggregation kinetics are discussed.

3.4.1 AF4 Optimization

The applicable size range for AF4 is several nanometers to tens of micrometers[14, 102, 104] and spans two modes or separation mechanisms. Fractionation of particles with sizes below 1 $\mu$m occurs in the normal mode and above 1 $\mu$m occurs in the steric mode.[95, 135, 136] The mode that dominates is determined by the magnitude of the particle diffusion ($D$) relative to its hydrodynamic diameter ($d_h$).[135, 136] Analytes smaller than 1 $\mu$m have $D$ values that significantly affect their equilibrium distances from the AF4 channel wall. In this normal mode, high $D$ (or small size) analytes diffuse farther into the channel than low $D$ analytes and elute first. For analytes larger than 1 $\mu$m, $D$ is relatively small compared to the analyte size and the elution is dependent on the location of the particles center of mass in the parabolic flow profile. In this steric mode, larger particles protrude farther into the channel, experience faster flows and elute before the smaller particles.
The cross over between normal and steric modes or the steric transition point is dependent on the channel dimensions, field strength and flow rates, and can be shifted between 0.3 and 3 \( \mu m \).\[38, 39\] Identifying the diameter at which the steric transition occurs is important for interpreting AF4 results and ensuring that coelution of two different sizes has not occurred. However, coelution may occur if the sample consists of components smaller than 0.3 \( \mu m \) and larger than 3 \( \mu m \). This situation can be resolved by pre-separating the sample into two fractions, one with component sizes that elute in normal mode and the other with components that elute in steric mode.\[137\] For this work, we circumvented this issue by using heat stress conditions that primarily produced aggregates in the nanometer and submicron size range.

The steric transition size was determined experimentally using polystyrene latex (PSL) standards (Figure 3.2). Example AF4 fractograms of PSL standards separated in the normal and steric modes are shown in Figure 3.2(a) and Figure 3.2(b), respectively. The retention times \((t_r)\) at the peak maxima of the PSL standards shown in Figure 3.2(a) and Figure 3.2(b) and additional individually injected PSL standards (not shown) are plotted versus diameter \((d)\) in Figure 3.2(c). Lines of best fit were applied to the normal mode (0.02 to 0.97 \( \mu m \)) and steric mode (3.0 to 20 \( \mu m \)) elution and their intersection places the steric transition at 1.2 \( \mu m \) thus allowing the entire 0.1 to 1 \( \mu m \) range of aggregates to be separated in the normal mode. These lines of best fit are used to determine protein aggregate sizes from their measured \( t_r \)s. These sizes (normal mode and steric mode) have a relative standard deviation of 7% or less.

Highly polydisperse samples such as submicron protein aggregates have continuous size distributions and AF4 separations will not yield multiple peaks, but rather a single broad peak. For these polydisperse samples, the size-based selectivity \((S_d)\) is a more meaningful metric to characterize the separation performance. The value for \( S_d \) is determined as the absolute value of \( d(\log t_r)/d(\log d) \).\[95\] The slopes of the best fit lines in Figure 3.2(c) yielded \( S_d \) values of 0.55 and 1.0 for normal and steric mode, respectively. The \( S_d \) for normal mode AF4 typically varies between 0.5 and 1.0. The relatively low normal mode AF4 \( S_d \) for this
Figure 3.2: Example AF4 fractogram for submicron PSL particles (solid line) and the $\dot{V}_c$ gradient program (dotted line) (a), micrometer PSL particles (b) and steric transition plot (c). The shaded area indicates the size region where elution mode changes from normal to steric mode. Solid lines are the best fits for normal mode and steric mode elution orders.
work was due to the $\dot{V}_c$ gradient program used to target the wide size range. However, this $S_d$ is higher than the 0.1 value typically observed for SEC.[19, 95]

3.4.2 AF4 of Anti-SA IgG Aggregates

Figure 3.3: AF4-UV-MALS analysis of uncentrifuged HS-AntiSA (10 min, 67°C). The left y-axis represents the UV response at 280 nm (solid line) and the LS 90° response (dashed line) and the right y-axis represents the $M$ determined by MALS (squares).

Heat is chosen as the stress method because significant amounts of submicron aggregates are created and this is the targeted size range for this study.[11, 36, 59] Figure 3.3 shows an example AF4 fractogram for an anti-SA sample with 10 minutes of heat stress at 67°C. No visible aggregates are present under this stress condition. The UV and light scattering at 90° (LS90°) signals and the $M$s determined by MALS are shown superimposed. The largest peak at 8 minutes with a flat M profile at $1.47 \times 10^5$ g/mol is attributed to anti-SA monomer. The broad peak from 9 to 18 minutes has a wide M distribution ranging from $2 \times 10^5$ g/mol to $>1 \times 10^6$ g/mol and is ascribed to aggregates. This tr range corresponds to 20 to 100 nm (calculated using PSL calibration plot in Figure 3.2). Between 6 and 15 minutes, MALS results confirm normal mode separation. The presence of a small void peak and large $M$s at 4.5 minutes suggests the elution of large, unretained material. Based on PSL standards
(Figure 3.2), these species are larger than 20 µm and eluted in steric mode. Accurate sizes and $M$s for these larger species cannot be reliably characterized by MALS[133], but are shown to emphasize their presence. These large sample components are discussed further in sections 3.3.3 and 3.3.4.

![Fractogram](image.png)

Figure 3.4: Fractogram of an IgG protein purchased from Sigma Aldrich showing the normalized UV signal (solid line) and the $M$s (filled squares). The $\dot{V}_c$ is 2.0 mL/min with a 10 minute linear gradient to 0.1 mL/min. The $\dot{V}_{out}$ is 0.5 mL/min.

Thus far, AF4 conditions are chosen to increase sample recovery, minimize the probability of shear degrading weakly bound aggregates, and separate the entire submicron size analytes in the normal mode. However, these conditions resulted in incomplete resolution of the monomer and aggregate species. To confirm that this incomplete resolution does not significantly affect the quantification of the monomer fraction $m$, the cross-flow rate was increased such that the monomer and aggregate species were resolved (Figure 3.4). A commercial IgG sample was used in Figure 3.4 because the significant amounts of dimer and trimer species allowed subsequent comparison of $m$ values for both cases of complete (0.68) and incomplete (0.69) resolution of monomer and aggregates. A two-tailed t-test confirmed that these $m$ values are not significantly different at a 95% confidence level. It should be
noted that using higher AF4 flow rates (Figure 3.4) to achieve better resolution may reduce sample recovery.[138]

3.4.3 Effects of Anti-SA IgG Sample Preparation Using Centrifugation

Centrifugation is commonly used in preparation of biological samples and serves to remove large components that may interfere with subsequent analyses and chromatographic separations. Heat stressed anti-SA IgG samples were centrifuged for 10 minutes at $9,900\,g$ to remove large aggregate species. These conditions are similar to those used in published work where submicron species remained in the sample solution after centrifugation at $>10,000\,g$ for 15 minutes.[36]

![Figure 3.5: Example LS 90° fractograms and $M_s$ of centrifuged (solid line) and uncentrifuged (dashed line) anti-SA (67°C, 20 min). The squares and triangles correspond to the centrifuged and uncentrifuged $M$, respectively.](image)

The broad size range of AF4 allows examination of the effects of the centrifugation step. The LS90° and $M$ plots for 20 minutes heat stressed anti-SA samples with and without centrifugation are shown in Figure 3.5. No significant change in the LS90° intensity or the $M_s$ is observed over the monomer peak indicating that the protein monomer is not affected by centrifugation and that there is no measurable aggregate co-elution with the monomer.
This observation applies to both centrifuged and uncentrifuged samples. However, the former shows significantly lower LS90° signal and Ms for the aggregate and void peaks compared to the uncentrifuged sample. The decrease over the aggregate peak (9 to 25 minutes) may be due to the removal of some submicron aggregates and the decrease over the void peak (4.5 minutes) is likely due to the removal of large (>20 µm) materials. While AF4 can separate these micrometer size aggregates, the Ms determined by MALS can be erroneously high.[134]

3.4.4 Heat Stressed anti-SA IgG Aggregate Formation

![Figure 3.6: LS 90° fractograms (lines) and Ms (shapes) for uncentrifuged (a) and centrifuged (b) heat stressed anti-SA.](image)

Figure 3.6: LS 90° fractograms (lines) and Ms (shapes) for uncentrifuged (a) and centrifuged (b) heat stressed anti-SA.

Figure 3.6 shows the growth of aggregates under different heat stress times with and without centrifugation. Both samples show a decrease in the monomer peak (8 minutes) and an increase in the aggregate peak (9 to 12 minutes) as the stress time is increased from 0 to 5 minutes. As the stress time is increased to 10 and 13 minutes, the aggregate peak intensity increases and extends to retention times of 23 minutes (~200 nm). At stress times >12.5 minutes, the solution became turbid and the aggregate peak intensity decreased while the intensity of void peak increased (Figure 3.6(a)). Results of centrifuged samples all showed
measurable removal of aggregate species eluting between 9 and 20 minutes and in the void peak (Figure 3.6(b)). The UV and LS90° signals of the centrifuged sample void peak were too weak for $M_s$ to be measured. Table 5.1 summarizes the $M^{agg}$ and $M^{tot}$ for the centrifuged and uncentrifuged anti-SA IgG1 samples. Both sets of data increase with increasing stress time. However, beyond 20 to 25 minutes of heat stress, $M^{agg}$ and $M^{tot}$ decrease. This is due to the formation of large particles that sediment to the bottom of the vial.

Table 3.1: Weight average molar masses for heat stressed anti-SA IgG1.

| Stress Time (min) | Centrifuged | | | | | | Uncentrifuged | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $M^{agg} \times 10^5$ (g/mol) | $M^{tot} \times 10^5$ (g/mol) | | | | $M^{agg} \times 10^5$ (g/mol) | $M^{tot} \times 10^5$ (g/mol) | | |
| 0 | - | 1.5 | - | 1.6 |
| 2.5 | - | 2.5 | 1.8 | 2.1 |
| 5 | 2.5 | 3.2 | 4.4 | 4.6 |
| 7.5 | 4.0 | 3.0 | 5.7 | 4.5 |
| 10 | 6.9 | 3.9 | 8.4 | 5.6 |
| 12.5 | 10 | 5.7 | 12 | 10 |
| 15 | 11 | 8.2 | 18 | 29 |
| 20 | 8.2 | 7.8 | 25 | 160 |
| 25 | 12 | 7.2 | 8.1 | 60 |
| 30 | 3.9 | 5.3 | 110* | 100* |

*Weight average molar masses determined at a stress time of 33 minutes.

Multiple injections of all heat stress samples were performed to ensure the repeatability of the AF4 method. For heat stressed anti-SA separations the total peak area showed less than 3 percent relative standard deviation for stressed samples without visible aggregation (<10 minutes of heat stress) and less than 5 percent relative standard deviation for stressed samples with visible aggregation (>10 minutes of heat stress). Sample recoveries were > 75% for unstressed and stressed (<10 min) anti-SA IgG1 samples. Estimates were not possible for stressed (>10 min) samples due to the loss by sedimentation of large aggregates. The relative standard deviations for the area under the aggregate peaks were 4-12%.
3.4.5 Determination of anti-SA IgG Aggregation Kinetics

The aggregation kinetics of heat stressed anti-SA samples at pH 6.25 were determined from AF4 peak areas, Ms from MALS, and Eqns. 3.1-3.3. The monomer fraction ($m$) loss and aggregate size ($M^{agg}/M^{mon}$) growth of heat stressed anti-SA are shown in Figure 6. The stress time, $t$, is scaled to $t_{50}$, the time at which 50% monomer loss ($m$=0.5) occurs. Centrifuged and uncentrifuged heat stressed anti-SA results were used to determine the LENP model parameters ($x$, $\delta$, $\tau_n$, $\tau_g$, and $\tau_c$).

Figure 3.7: Centrifuged (black) and uncentrifuged (red) anti-SA IgG1 results for $m$ (filled symbols) and $M^{agg}/M^{mon}$ (open symbols) are plotted as function of time ($t$) scaled by the half life ($t^{50}$). The lines represent LENP model fits to $m$ (solid line) and $M^{agg}/M^{mon}$ (dashed line). Error bars are standard deviations with n=3.

Figure 3.7 shows the $m$ and $M^{agg}/M^{mon}$ for the centrifuged and uncentrifuged data and the corresponding fits determined by LENP model. For both samples, the $m$ decreases while the $M^{agg}/M^{mon}$ increases. However, the increase in $M^{agg}/M^{mon}$ is slower for the centrifuged sample. Regression of the solutions from Eqns. 3.1-3.3 to the centrifuged anti-SA $m$ and $M^{agg}/M^{mon}$ data showed good fits with $R^2 >0.98$ and $>0.94$, respectively. Data points after $t/t_{50} = 1.0$ were not included in the LENP model fitting because of significant aggregate
removal by centrifugation and precipitation. Solutions to Eqns. 3.1-3.3 were also regressed to the uncentrifuged $m$ and $M^{agg}/M^{mon}$ data with $R^2 > 0.98$ and $>0.94$, respectively. The $M$s were determined using only the aggregate peak eluting from 9 to 25 minutes. The large material (>20 µm) eluting in the void peak was not included in the uncentrifuged data due to the limitations of MALS. Data points for $M^{agg}/M^{mon}$ after $t/t_{50} > 1.33$ were not included in the fits due to aggregate phase separation. The cut off values of $t/t_{50} = 1.0$ and 1.33 for the centrifuged and uncentrifuged samples, respectively, were chosen because sample loss due to sedimentation will lead to inaccurate conclusions about the aggregation kinetics. The anomalously low $M^{agg}/M^{mon}$ values after $t/t_{50} = 1.0$ and 1.33 are included to show the effects of aggregate condensation and precipitation that occurs at long stress times.[130]

| Table 3.2: Kinetic parameters determined from the LENP model. |
|-----------------|-----|------|-----|-----|
| Sample          | $x$ | $\delta$ | $\tau_n$ (min) | $\tau_g$ (min) | $\tau_c$ (min) |
| Centrifuged     | 3   | 1     | 7   | 0.09 | 0.025 |
| Uncentrifuged   | 3   | 1     | 12  | 0.05 | 0.020 |

Table 3.2 lists the values of $x$, $\delta$, $\tau_n$, $\tau_g$, and $\tau_c$ determined for the fits shown in Figure 3.7. In agreement with empirical observations made by Li et al., the fits were found to be relatively insensitive to changes in $x$ and sensitive to changes in $\delta$.[129] Small changes in the $\tau_n$, $\tau_g$, and $\tau_c$ values (Table 3.2) were observed between the uncentrifuged and centrifuged samples. Both samples have a relatively large $\tau_n$ compared to the smaller $\tau_g$ and $\tau_c$ time scales indicating slow nucleation relative to aggregate growth. The $\tau_g$ and $\tau_c$ values are on the same order of magnitude suggesting that chain polymerization and aggregate condensation growth are competing mechanisms. The $\tau_n$, $\tau_g$, and $\tau_c$ values determined for heat stressed anti-SA indicate an aggregation mechanism that is characterized by slow nucleation with appreciable aggregate condensation and phase separation at longer stress times. This is in agreement with other work where significant condensation and subsequent precipitation is expected for samples at pH near the protein pI due to reduced charge repulsion between aggregates.[59]
Centrifugation does not significantly affect the anti-SA IgG1 aggregation mechanism under conditions employed in this work. However, removal of aggregates by centrifugation decreases the measured $M$s and has the potential to influence the kinetic mechanism.

Figure 3.8: Plots of $M_{\text{tot}}/M_{\text{mon}}$ versus extent of reaction $(1-m)^2$ for centrifuged (black squares) and uncentrifuged (red triangles). Dotted lines are guides to the eye.

AF4s ability to separate large (> 20 µm) and monomer species allow qualitative kinetic analyses over a broad size range. Plotting $M_{\text{tot}}/M_{\text{mon}}$ versus the extent of reaction, $(1-m)^2$ is useful for qualitatively determining differences in the aggregation mechanisms.[128] Figure 3.8 shows superimposed $M_{\text{tot}}/M_{\text{mon}}$ vs. $(1-m)^2$ plots for the centrifuged and uncentrifuged samples. The latter show higher $M_{\text{tot}}/M_{\text{mon}}$ over the entire $(1-m)^2$ range suggesting that large aggregate species form even at early stress times. The centrifuged sample shows a slight concave up trend at $(1-m)^2 < 0.25$ signifying aggregate condensation.[59, 128] The decrease in $M_{\text{tot}}/M_{\text{mon}}$ at $(1-m)^2 > 0.25$ is attributed to aggregate phase separation. The uncentrifuged sample shows a steep upturn at $(1-m)^2 < 0.25$ signifying aggregate condensation. These results suggest that phase separation and aggregate condensation occur at lower reaction extents than that for the centrifuged sample. This qualitative comparison of the centrifuged and uncentrifuged data provides additional insights into the formation of
micrometer-sized and visible particles, even at early stress times, and the potential effects of removing sample species by centrifugation.

AF4 allows for the separation of large aggregates up to and larger than 1 m. In SEC based kinetic analyses, these species are typically treated as unreactive (no longer participating in aggregation) because they precipitate or are removed by centrifugation. The removed aggregates may still participate in aggregate condensation growth and the LENP model can be used to determine their aggregation kinetics. Unfortunately, MALS does not allow accurate determination of $M$ for these large aggregate species. Other detection methods such as flow microscopy, light obscuration, or laser diffraction are needed to quantitatively measure the size of these large aggregates and will be the focus of future aggregation kinetic studies.

3.5 Conclusion

Understanding aggregate formation is important for controlling or preventing aggregation in therapeutic formulations. A simple asymmetric flow field-flow fractionation method was successfully developed to fractionate submicron aggregates in the normal mode and characterize nanometer and submicron aggregation kinetics. Fitting the LENP model to centrifuged and uncentrifuged heat stressed anti-SA IgG1 samples yielded a potential mechanism involving slow nucleation and aggregate-aggregate condensation. While centrifugation did not alter the kinetic mechanism, this may not hold true for other experimental conditions. Qualitative analysis of centrifuged and uncentrifuged HS anti-SA results yielded insights into the formation of larger aggregate species at early times and showed the applicability of AF4 for the analysis of micrometer aggregate species. AF4’s ability to fractionate aggregates up to and larger than 1 $\mu$m is advantageous for kinetic studies where aggregates larger than 100 nm may not be well retained by SEC or easily characterized by other methods.
CHAPTER 4
IMPACT OF ASYMMETRICAL FLOW FIELD-FLOW FRACTIONATION ON PROTEIN AGGREGATES STABILITY

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4.1 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 shear stress (related to sample injection), carrier liquid buffer, 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 the carrier liquid buffer system is important 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

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rapidly dissociate (<∼ 2 seconds) upon dilution as the size calculated by AF4 theory may be more accurate than that measured by online DLS. Experimentally, the $d_h$ 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.

4.2 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.[14] 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 SECs applicability.[19, 45, 115, 139–141]

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.[31] As shown in Figure 4.1, 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 cross-flow ($\dot{V}_c$) 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 ($d_h$).[142] Equation 4.1 shows the relationship between,
Figure 4.1: Schematic of the AF4 channel showing processes during analysis.

and $D$ for retention ratios $<0.2$

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

(4.1)

where $w$ is the channel thickness. In many cases, AF4 also allows greater flexibility in carrier fluid choice compared to SEC [37] and is able to separate over a wide size range (0.01 to $>1 \mu$m) with good selectivity (>0.5)[143]. These advantages are at the heart of many publications that provide insights into the formation and dissociation of biological and synthetic complexes and aggregates.[28, 34, 41, 144] AF4 determination of dissociation constants ($K_d$s) has allowed the true stoichiometric binding of protein-protein[28] and DNA-protein[34] complexes to be assessed in solution at $<\mu$M affinities. Detection of weakly bound aggregate species can also be achieved by adjusting AF4 conditions.[41] The dissociation of polymer complexes and micelles loaded with fluorescent dyes or nanoparticles has been monitored by AF4.[90, 144] 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 [145–147], there have been concerns that several steps during the AF4 process may affect delicate
or weakly bound protein aggregates species[14, 115]. 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[148], administration using pre-filled syringes[149], and flow in physiological systems[150] because of the potential for induced 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.[148, 151, 152] Syringes with 22 gauge (0.413 mm inner diameter) needles are typically used to load samples into the injection valve (Figure 4.1, Inset a). 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 Figure 4.1, Inset b. Fluid exits the channel through the semi-permeable membrane wall thereby simultaneously providing a cross-flow 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.[95] Typically, sample concentrations at the accumulation wall are 10 to 100 times greater than
that of the original sample. 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 an FFF channel and online concentration has been used to analyze colloids in environmental suspensions, polystyrene latex beads (PSL), and proteins. However, the focusing step can also lead to sample aggregation and/or increased membrane interactions if excessively long times are used. The sample focusing step has also been used as an “online incubation” to investigate IgE-aptamer binding. 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, but this subject is beyond the scope of this work and has been summarized elsewhere. 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 4.1, Inset c). Shear and dilution are inherent to separation techniques and may play a role in dissociation of aggregates as previously shown in SEC and cation exchange chromatography studies. Aggregates can completely dissociate, partially dissociate, or remain intact depending on the timescale of the separation relative to the rate of dissociation. Shear degradation in SEC columns has been reported for protein aggregates and a variety of high $M$ polymers while shear stress during thermal field-flow fractionation (ThFFF) analysis has shown no evidence of polymer degradation. Typically, shear rates in FFF channels ($\sim 1$ – $20$ s$^{-1}$) are orders of magnitude lower than in SEC columns ($\sim 1000$ – $10000$ s$^{-1}$). AF4 without focusing and cross-flow has been used to study loosely bound aggregates and showed that these species remained intact during analysis. 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[21]. 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[169], 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[21], 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 4.1, Inset d). 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[37, 46] 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 $M$.[170] 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.

4.3 Materials and Methods

Purified anti-streptavidin (anti-SA) IgG1 was provided by Amgen (Thousand Oaks, CA)
in formulation buffer at $\sim 30$ mg/mL. $NaH_2PO_4 \cdot H_2O$, $Na_2HPO_4$ (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.

### 4.3.1 Sample and Carrier Fluid Preparation

All carrier fluid buffers are prepared using distilled deionized (DI) 18.2 MΩ water (APS Filtration AquaMax Ultra, Nan Nuys, CA). Phosphate buffer (100 mM, pH 6.25) is prepared by mixing 10.99 g of $NaH_2PO_4 \cdot H_2O$, 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 $C_6H_8O_7H_2O$ 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 4.2.2. 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.

### 4.3.2 Asymmetrical Flow Field-Flow Fractionation and Multi-Angle Light Scattering or Dynamic Light Scattering

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 µm 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.

Table 4.1: AF4 Conditions for the Preparation and Re-analysis of Aggregates Samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample Loop Volume</th>
<th>Focusing Flow Rates (inlet; focus)</th>
<th>Focusing Time</th>
<th>Separation Flow Rates (cross-flow; outlet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregate Sample</td>
<td>100 µL</td>
<td>0.2 mL/min; 3 min</td>
<td>1.0 mL/min to 0.1 mL/min</td>
<td></td>
</tr>
<tr>
<td>Preparation</td>
<td></td>
<td>1.3 mL/min</td>
<td>over 10 min; 0.5 mL/min</td>
<td></td>
</tr>
<tr>
<td>Aggregate Sample</td>
<td>1.25 mL</td>
<td>0.2 mL/min; 7.2, 9.2, 11.2, 13.2 min</td>
<td>1.0 mL/min to 0.1 mL/min</td>
<td></td>
</tr>
<tr>
<td>Re-analysis</td>
<td></td>
<td>1.3 mL/min; 13.2 min</td>
<td>over 10 min; 0.5 mL/min</td>
<td></td>
</tr>
</tbody>
</table>

Aggregate sample preparations and subsequent PBS and CA aggregate sample analyses were run under the conditions shown in Table 5.1 unless specified otherwise. Separations where AF4 sizes were calculated from retention theory used constant outlet and cross-flow 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.

4.3.3 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.
4.3.4 Batch Mode Dynamic Light Scattering

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

4.4 Results and Discussion

The impacts of AF4 on protein aggregate stability are discussed below.

4.4.1 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.[143, 171] Two commonly used buffers, phosphate and citric acid, are selected to allow comparison of aggregates stability using different 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 4.2. In both buffers, a large monomer peak with a uniform $M$ of $1.5 \times 10^5$ g/mol is observed at $\sim 5$ minutes. The PBS preparation fractogram (Figure 4.2a) also has a broad peak from 6 to 20 minutes with an $M$ ranging from $2.0 \times 10^5$ to $1.0 \times 10^7$ 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 may contain large unretained material eluting in the steric mode or small fragments of the monomer formed during the heat stress procedure.[172] Aggregate samples are also prepared in a pH 5.0, 10 mM citric acid buffer with 500 mM NaCl. This CA buffer was selected because it has been shown to form large and stable anti-SA IgG1 aggregates when subjected to heat stress.[171] Figure 4.2b 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 ($3 \times 10^5$ to $2 \times 10^7$ g/mol) are present.
Figure 4.2: Fractograms (line) and Ms (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.
4.4.2 Effect of Carrier Fluid on Protein Aggregate Stability

The importance of buffer type 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 4.3) 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 4.4a) 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 4.4b).

Figure 4.3: Fractograms of anti-SA IgG1 suspended in citric acid buffer. The solid line and filled circles represents the unstressed Anti-SA IgG1 and the shaded area represents the collection of the monomer fraction (CA-M). The dashed line and open circles represent the re-analysis of the collected CA-M fraction.
Figure 4.4: a) Fractograms (line) and Ms (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.

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 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 observed increase in $M$ from 6 to 20 minutes for CA-A2 is due to aggregates that range from oligomers to large species $>2 \times 10^6$ g/mol. The measured $Ms$ 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 the role buffer type plays in aggregate stability during AF4 analysis.

4.4.3 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.[148, 149] 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 [173], 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 $M_s$ (Figure 4.5b). 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 are shown in Figure 4.6.

![Fractograms](image)

(a) and (b)

Figure 4.5: Fractograms (lines) and molar masses (symbols) for analysis of a) CA-A1 and b) CA-A2 unstressed and after syringe stress (SG1X and SG3X). RT30 represents incubation of CA-A1 and CA-A2 at room temperature for 30 minutes before re-analysis.
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.

4.4.4 Effect of Sample Focusing Time on Aggregates Stability

Sample focusing plays an important role in improving retention and resolution in AF4 separations.[153, 157] Ideally, the focusing time is optimized to reduce potential analyte-analyte and analyte-membrane interactions[44] 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.[21, 167, 174, 175]
Figure 4.7 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 4.7a). The higher void peak areas for shorter focusing times are likely due to incomplete sample relaxation.[95, 176] 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 ($\sim 1$ min for 7.2 minutes focusing, $\sim 7$ minutes for 13.2 minutes focusing) and may not be fully relaxed. Figure 4.7b also shows an increase in the void peak for CA-A2 as the focusing time decreases because of incomplete relaxation. Fluctuations in the monomer and aggregate peak intensities are primarily due to differences in the prepared CA-A2 sample amounts. However, the sample recoveries for each focusing time are not significantly different and did not impact the results or conclusions drawn.

The weight average $M$ of the aggregate peak and the percent monomer and percent aggregates are calculated for each focusing time (Figure 4.8). As expected, the CA-A1 $M_w$ is lower than CA-A2 for all focusing times (Figure 4.8a). No significant difference between
average $M_s$ is observed with increasing focusing time for either aggregates sample. Similarly, no significant difference is observed in the percent monomer with increasing focusing time for either sample (Figure 4.8b). Increasing the focusing time from 9.2 to 13.2 minutes did not significantly change the percent aggregate present in CA-A1 or CA-A2. However, comparison of a 7.2 minute focusing time to 13.2 minutes showed a significantly different percent aggregates for both fractions. This difference is most likely due to insufficient relaxation and not a change in the aggregate population.

![Graph](a)  
(a) The average $M_s$ and 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.

![Graph](b)  
(b)

In summary, increasing focusing time did not significantly change the average $M$ or percent monomer or percent aggregate for CA-A1 or CA-A2. This suggests that the focusing step alone does not significantly change the heat stressed anti-SA IgG1 aggregates under the separation conditions used in this work. However, aggregates formed under different stress types and solution conditions may show a different susceptibility to dissociation.[172]

It is recommended that the potential impacts of focusing be investigated as part of the AF4 method development for individual different proteins, stress treatments, and solution conditions.
4.4.5 Effect of AF4 Separation on Aggregates Dissociation

During separations of protein aggregates, there is the potential for dissociation in the channel.[21] Table 4.2 shows a comparison of $t_r$, 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 $\sim 70\%$ 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 4.9a 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 μm from the accumulation wall.[153] This represents only ~3% of the channel thickness or ~6% of the channel volume. Near the beginning of the channel, nonequilibrium is the major zone spreading mechanism.[177] 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.[178]

Table 4.2: Comparison of CA-A1 and CA-A2 Original and Re-analyzed Samples.

<table>
<thead>
<tr>
<th></th>
<th>CA-A1</th>
<th>CA-A2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Original</td>
<td>Re-analysis</td>
</tr>
<tr>
<td>$t_r$ (min)</td>
<td>8.5*</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>$M_w$ ($\times 10^6$ g/mol)</td>
<td>1.35 ± 0.08</td>
<td>1.11 ± 0.10</td>
</tr>
<tr>
<td>PDI</td>
<td>1.14 ± 0.01</td>
<td>1.30 ± 0.05</td>
</tr>
<tr>
<td>Monomer (%)**</td>
<td>-</td>
<td>25.0 ± 2.1</td>
</tr>
<tr>
<td>Aggregate (%)**</td>
<td>-</td>
<td>68 ± 4</td>
</tr>
</tbody>
</table>

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.

Figure 4.9b shows the calculated concentration at the zone center at each position, z, along the channel length, $L$, for a 100 μL injection of a 1 mg/mL anti-SA IgG1 sample and a 27.6 cm long channel. Calculations are performed as previously shown by Litzn.[169] 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 ($c_\infty$) initially decreases to ~0.75 mg/mL and then increases to ~1.25 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 ~0.25 mg/mL in the first half of the channel to ~0.5 mg/mL at the end of the channel. These $c_l$ values represent an average sample dilution of ~2-4× during the separation. The maximum dilution occurs at $z \sim 10$ cm with an average
Figure 4.9: 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.
sample concentration of ∼0.25 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).[153] Figure 4.9c shows the concentration distribution as a function of distance from the accumulation wall and nonequilibrium band broadening $\sigma$ (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 μm 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 20× to 100× in SEC depending on the column and separation conditions compared to the 2× to 4× 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 4.1, Inset d) just prior to eluting. The outlet sample concentration ($c$) can be calculated using Equation 4.2[179]

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

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 $H$ 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 ∼20× 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).

![Fractograms for heat stressed anti-SA IgG1](image)

Figure 4.10: 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 $d_h$s determined by online DLS and AF4 theory, respectively.

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 4.10 shows the AF4-DLS fractograms of heat stressed anti-SA IgG1 obtained using different absolute cross-flow and channel outlet flow rates while holding the ratio of $\dot{V}_c/\dot{V}_{out}$ constant at 2. Under such condi-
tions, 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 on-line DLS measurements and AF4 theory. Lower resolution is expected at lower absolute flow rates[179] 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 \( \sim 4 \) 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.

4.5 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 $\sim 2-4 \times$ and that the majority of the AF4 dilution ($\sim 20 \times$) 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).

4.6 Acknowledgments

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CHAPTER 5
COVALENT MODIFICATION OF ULTRAFILTRATION MEMBRANES FOR ASYMMETRICAL FLOW FIELD-FLOW FRACTIONATION

In this work, a converted flow field-flow fractionation (FlFFF) channel is used as a reactor to enable the modification of large ultrafiltration (UF) membrane areas while keeping the surface smooth and free from creases. The potential for unwanted analyte-membrane interactions is a major limitation for FlFFF techniques, but can be prevented by covalently modifying the surface with antifouling materials. However, several challenges have hindered the modification of UF membranes for use in FlFFF: 1) large membrane areas must be modified (∼90-100 cm²) and 2) the membrane surface must remain smooth and flat. We have developed a method to graft poly(N-isopropylacrylamide) (PNIPAM) brushes from regenerated cellulose ultrafiltration (UF) membranes with large, flat surface areas. Atom transfer radical polymerization (ATRP) was selected to graft PNIPAM from the membrane surface and the reaction mixture was re-circulated through the channel reactor to facilitate even membrane coverage. Fourier transform infrared (FTIR) spectroscopy before and after ATRP confirmed the polymerization of PNIPAM on the surface of the regenerated cellulose membrane. Contact angle measurements indicated a change in membrane hydrophobicity further supporting successful polymer grafting. Water flux measurements were not consistent between different membrane pieces suggesting that differences in porosity exist over from area to area of the membrane. Modified membranes were tested in FlFFF channels and yielded successful fractionation of IVIg and BSA proteins. Additionally, FlFFF analysis above the lower solution critical temperature (∼32°C) resulted in lower protein recovery indicating that membrane hydrophilicity can be controlled. Further optimization is still needed, but we believe this method will provide the necessary groundwork for future advances in FlFFF membrane modification.
5.1 Introduction

Ultrafiltration (UF) membranes are widely used for wastewater treatment, drinking water purification, food preparation, and bioprocessing.\textsuperscript{[180–184]} The small pore sizes (1-100 nm) of UF membranes make them suitable for separations of nano-scale materials. In particular, UF membranes are frequently used in biotechnology processes including protein concentration and buffer purification.\textsuperscript{[184–186]} Despite widespread use, protein fouling remains a major challenge that limits UF membrane performance.\textsuperscript{[187]} Adsorption of proteins to the membrane surface can result in a buildup of material causing a loss of both material and membrane permeability. Cleaning is often necessary to restore the membrane function\textsuperscript{[181, 188]}, but ideally membrane fouling can be prevented by modifying the surface to reduce unwanted analyte interactions.\textsuperscript{[189]}

In addition to their use in industrial applications, several analytical techniques also employ UF membranes including asymmetrical flow field-flow fractionation (AF4). The UF membrane, frequently regenerated cellulose (RC), is one of the key features of the AF4 channel that enables the separation of nanoparticles, proteins, and polymers. Several studies have also shown the applicability of using AF4 to quantitatively evaluate membrane fouling.\textsuperscript{[158, 190]} The AF4 separation takes place in a ribbon-like channel formed by thin trapezoidal spacer sandwiched between a clear top wall and a semi-permeable accumulation wall. Typically, the channel is 100 to 500 $\mu$m thick, 20 to 30 cm in long, and 2 to 3 cm wide. The accumulation wall is composed of porous frit covered with a semi-permeable UF membrane which allows some carrier fluid to exit the channel. This flow forces analytes towards the accumulation wall and diffusion allows them to migrate back up into the channel. At equilibrium, analytes occupy an average height in the channel on the order of $\sim 10$ $\mu$m. Simultaneously, a parabolic flow profile formed across the channel thickness is pumped down the channel length with the slowest flow velocities near the channel walls and the highest velocities in the channel center. Large analytes with small diffusion coefficients experience slower velocities near the walls and elute after smaller analytes with large diffusion coeffi-
cients that feel faster velocities. Based on the elution time of the analytes, the diffusion
coefficient and in turn the hydrodynamic size can be determined.[95, 179]

Ideally, the membrane would have no unwanted analytes interactions, allow for com-
plete sample recovery, and not influence the measured elution time.[44] Selecting the best
membrane and solution conditions to reduce analyte-membrane interactions is critical for
obtaining good separations. However, the potential for these interactions remains one of
the major limitations of the AF4 technique.[191, 192] Therefore, membrane modification to
reduce fouling could address this key challenge.

Chemical modification of UF membranes with polymers is a promising method to impart
antifouling properties. The van-der Waals, electrostatic, and hydrophobic forces between pro-
teins and membranes can be controlled and tuned using surface initiated polymerization.[193]
Additionally, polymer modification can introduce new chemical functionality or stimuli-
responsive properties.[194, 195] A grafting-from UV-initiated approach has been used for
surface modification.[196–199] Photochemical approaches have been applied to a variety of
membrane and polymer chemistries with many methods developed for poly(ether sulfone)
(PES) and polysulfone (PS) membranes.[193] Plasma-induced graft polymerization has also
been used for the modification of membrane surfaces.[200–202] Modifications by plasma
polymerization have shown reduced protein fouling and low organic fouling in wastewater
applications.[180, 201, 203] Additional surface polymerization methods to reduce membrane
fouling include redox-initiated and enzymatic grafting approaches among others.[204, 205]

Recently, atom transfer radical polymerization (ATRP) has been used for membrane
surface modification.[206] In ATRP, an equilibrium is formed between the initiator, typically
an alkyl halide, and the radical species.[207] The initiator is first attached to the membrane
surface and a transition metal catalyst generates radicals, so polymer growth occurs at the
membrane surface. This allows for formation of polymers with controlled molecular weight
and polydispersity attached to the membrane surface. Additionally, high grafting densities
can be obtained by using a grafting from approach in contrast to a grafting to approach.[208]
ATRP has been used to modify a variety of membrane types [209–211] by grafting polymers from the membrane surface.[206] Specifically, regenerated cellulose (RC) membranes have been modified by ATRP using poly(2-dimethylaminoethyl methacrylate) (poly(DMAEMA)) [211], poly[poly(ethylene glycol) methacrylate] (PPEGMA) [212], poly(N-isopropylacrylamide) (PNIPAM)-block-poly(oligoethylene glycol methacrylate) (PPEGMA) [213], and PNIPAM [214]. In particular, stimuli-responsive polymer brushes allow tuning of the membranes surface properties. The use of PNIPAM as a protein-resistant polymer brush with thermo-responsive properties has been used to modify membranes for a variety of applications. PNIPAM exhibits a lower critical solution temperature (LCST) of \(\sim 30-35^\circ\text{C}\) that allows for relatively easy temperature control of its properties (Figure 5.1). PNIPAM is in an extended, hydrophilic conformation below the LCST and is in a collapsed, more hydrophobic conformation above the LCST. This change in hydrophilicity is useful for controlling protein-membrane interactions because these interactions are thought to be due to the exposure of hydrophobic protein residues. Several studies have shown that protein-surface interactions increase above the LCST and are reduced below the LCST.

**Below LCST (<32°C)**
- Extended Hydrophilic Conformation
- Regenerated Cellulose Membrane

**Above LCST (>32°C)**
- Collapsed More Hydrophobic Conformation
- Regenerated Cellulose Membrane

![Figure 5.1: Illustration of the conformation change at the LCST of PNIPAM.](image)

Currently, grafting of polymer brushes from membrane surface is done on small, commercially available RC membranes (<4.7 cm in diameter) with surface areas <20 cm\(^2\). However, AF4 uses long rectangular pieces of flat sheet membranes (~3x30 cm) as the channel accumulation wall. Additionally, these membranes must be smooth, flat, free from creases, and
the flux cannot be significantly decreased to avoid perturbations during the separation. This poses a significant challenge for modifying AF4 membranes when coupled with potentially large amount of reagents required to modify such a large area. Therefore, new scaled up reactors to modify large membrane surface areas (>\(
\sim
\) 90 cm\(^2\)) that keep the membrane flat and reduce reagent volumes are needed. In this work, we develop a novel reactor capable of modifying large membrane areas (>3x30 cm) using ATRP. The reactor was designed to significantly reduce the amount of reagents need to carry out the modification and to keep the membrane surface flat and smooth for use in AF4. As a proof of principle, PNIPAM, known for its protein resistant and stimuli responsive properties, was grafted from the surface of a 30 kDa molecular weight cut-off (MWCO) RC membrane (Figure 5.2). The grafting of PNIPAM was confirmed by fourier transform infrared (FTIR) spectroscopy and captive bubble contact angle. The thermo-responsive properties and performance of the modified membranes were tested in an AF4 channel and yielded successful fractionations of IVgG and BSA protein samples above and below the PNIPAM LCST.

![Figure 5.2: Schematic of regenerated cellulose membrane modification.](image)

5.2 Materials and Methods

Regenerated cellulose ultrafiltration flat sheet membrane (30 kDa MWCO) on a polypropylene support was purchased from Microdyn-Nadir (Wiesbaden, Germany). The follow-
ing chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless stated otherwise: tris[2-(dimethylamino)ethyl]amine (Me6TREN), bromo isobutyryl bromide (98%), and ethyl 2-bromoisobutyrate (98%). Anhydrous isopropanol (>99.5%) and anhydrous dichloromethane (>99.7%) were purchased from Alfa Aesar (Haverhil, MA) and used as received. Copper (I) chloride (Sigma-Aldrich) was purified in acetic acid, and N-isopropylacrylamide (NIPAM) (Sigma-Aldrich) was recrystallized once in 1:1 hexane/toluene. Diethyl ether (99.9%), and hexanes were purchased from Fischer Scientific (Waltham, MA). Glacial acetic acid were purchased from Macron Fine Chemicals. Deuterated chloroform (99.8%) was purchased from Cambridge Isotope Laboratories, Inc. Methanol (99.9%) was purchased from Pharmco-AAPER. Lyophilized BSA was purchased from Sigma Aldrich. The IVIg protein was provided by the Center for Pharmaceutical Biotechnology (University of Colorado, Denver).

5.2.1 Surface-initiated ATRP on Small RC Membranes

Regenerated cellulose membrane (4 cm²) was purged of air under nitrogen for 15 min. Dry pyridine (0.08 mL, 1 mmol) and BIBB (0.12 mL, 1 mmol) were added via syringe, and the solution left at room temperature for 2 hours. The membrane was washed in water, THF, and isopropanol and left in isopropanol until the time of polymerization.

The initiator-functionalized membrane sample, N-isopropylacrylamide (1.13 g, 10 mmol) and Me6TREN (14 µL, 0.05 mmol) in isopropanol (4 mL), and CuCl (0.0049 g, 0.05 mmol) were placed in three separated schlenk flasks and purged under argon for 30 min. The NIPAM solution was transferred to the flask containing CuCl, and the ligand allowed to complex for 30 min. The initiator, ethyl 2-bromoisobutyrate (8 µL, 0.05 mmol) was added via microsyringe, and the solution immediately transferred to the schlenk flask containing the membrane. The solution was left overnight, and the reaction terminated by exposure to air. Polymer in solution was dissolved in THF and purified in a silica gel column, then precipitated into cold diethyl ether.
5.2.2 Surface-initiated ATRP on Large RC Membranes

Regenerated cellulose membranes with dimensions of 4.8 cm x 48.3 cm were prepared for large-scale modification. The membranes were placed in the large-scale channel reactor for initiation and subsequent polymerization. The channel was formed using a Teflon sheet with a thickness of 0.053 cm, a width of 4.8 cm, and a length of 48.3 cm and a 3.2 cm x 45.2 cm rectangle was cut from the middle to create a channel spacer. The membrane and Teflon spacer were pressed between two Teflon blocks. Two Plexiglas blocks were then used to bolt the membrane, Teflon spacer, and Teflon blocks together at 80 inch-pounds. This creates a channel with a total volume of 7.7 mL. At each end of the reactor channel two Teflon tubes (0.31 cm outer diameter, OD, and 0.16 cm inner diameter, ID) entered the upper Teflon block and were sealed with plastics super glue.

The channel was washed with dry isopropanol prior to initiation. Triethylamine (0.8 mL) and BIBB (1.4 mL) in dry isopropanol (20 mL) were injected into the channel via syringe, switching between inlet and outlet tubes every 10 mL. After 2 hours, the channel was rinsed by circulating 100 mL isopropanol and then purging the channel with 20 mL dry isopropanol.

A 3-neck flask was attached to the channel and a peristaltic pump using Accu-ratedTM PVC pump tubing (Elkay, United Kingdom) with an ID of 0.28 cm and an OD of 0.45 cm. Dry isopropanol (25 mL) was purged with nitrogen while pumping through the channel for 30 minutes. CuCl (10.8 mg) was weighed into a round bottom flask and purged with nitrogen for 30 minutes. Next, 9 mL of isopropanol from the 3-neck flask was transferred by cannula to the CuCl flask and 27 µL of tris[2-(dimethylamino)ethyl]amine (Me6TREN) was added and the flask was purged with nitrogen for 30 minutes. N-Isopropylacrylamide (NIPAM) (2.2523 g) was weighed into a third round bottom flask, 9 mL isopropanol (from 3-neck flask) was transferred by cannula and the flask was purged with nitrogen for 30 minutes. The CuCl/Me6TREN solution was transferred by cannula to the PNIPAM flask and the mixture was then transferred to the 3-neck flask attached to the channel reactor. Ethyl bromoisobutyrate (16 µL) was added to the reaction mixture and the peristaltic pump was
then turned on to circulate. While under nitrogen pressure, the reaction mixture circulated through the channel for 4.5 hours.

### 5.2.3 Fourier Transform Infrared Spectroscopy

ATR FT-IR (Thermo Scientific, Nicolet iS50, OMNIC software) was used to characterize the surface of unmodified, initiated, and PNIPAM-modified membranes. Membranes were rinsed in DI water and methanol and air-dried prior to analysis.

### 5.2.4 Contact Angle Measurements

The contact angle goniometer (Ram-hart Instrument Co., model 200-00, Succasunna, NJ) was used to measure the captive bubble contact angle. After washing in isopropanol and DI water, samples were clamped, with the modified surface facing downward, on an immersible stage, and submerged in 18 MΩ water. A 6 µL bubble was introduced underneath the membrane and static contact angles were determined using DROPimage Standard software (Ram-hart Instrument Co., Succasunna, NJ). Average contact angles and 95% confidence intervals were determined using at least 5 bubbles per sample. Contact angles were measured at 20°C and 40°C.

### 5.2.5 Asymmetrical Flow Field-Flow Fractionation

An AF2000 System (Postnova, Germany) coupled online to a SPD-20A UV/Vis detector (Shimadzu, Kyoto, Japan) was used to characterize unmodified and modified membrane performance and fouling. A 350 µm thick spacer was used to form a trapezoidal channel with a length of 28.8 cm and maximum width of 2 cm and minimum width of 0.5 cm. Flow rate conditions are given in the figure legend for each data set. All carrier fluids were prepared using 18 MΩ water (APS Filtration, Aquamax Ultra, Buffalo, NY). The IVIg protein samples were analyzed using 0.05 M phosphate buffer dissolving 2.670 g NaH₂PO₄ and 4.352 g Na₂HPO₄ in 1 L 18 MΩ water. The anti-SA IgG1 protein samples were prepared using a 0.01 M citric acid buffer with 0.5 M NaCl using 2.10 g of C₆H₈O₇H₂O and 29.22 g
of NaCl in 1 L 18 MΩ water.

5.3 Results and Discussion

First, the modification of small scale (∼4 cm$^2$) membranes is shown. Then, the synthesis is scaled up to modify large scale (∼90 cm$^2$) membranes for AF4.

5.3.1 Small-scale Membrane: Characterization

ATR-FTIR spectroscopy was used as to determine the chemical properties of the unmodified, BIBB initiated, and PNIPAM modified RC membrane surfaces (Figure 5.3). In ATR-FTIR the penetration depth of the IR beam is dependent on the refractive indices of the sample and ATR crystal as well as the wavelength and the incident angle of the IR beam.[215] This results in the highest sensitivity at the membrane surfaces because the intensity of the reflected wave decreases exponentially with increasing depth leading to lower sensitivity with increasing depth. The unmodified membrane has a large characteristic peak at 3310 cm$^{-1}$ representing the hydroxyl groups of the RC membrane and an alkane stretch at 2884 cm$^{-1}$. Both peaks decrease significantly after initiation with BIBB suggesting a successful surface initiation of the membrane surface via reaction of BIBB with surface hydroxyl groups (Figure 5.2). Additionally, the carbonyl stretch of the ester formed by the reaction between BIBB and the RC hydroxyl group appears at 1736 cm$^{-1}$ further supporting membrane initiation.

Upon surface polymerization of the membrane for increasing amounts of time the ATR-FTIR analysis shows the presence of several peaks from ∼1400 cm$^{-1}$ to 1700 cm$^{-1}$ (Figure 5.3). The peak at 1645 cm$^{-1}$ is attributed to the amide carbonyl stretch and the peak at 1535 cm$^{-1}$ is attributed to the bending mode of the amide group.[216] Bands at 1458 cm$^{-1}$ and 1367 cm$^{-1}$ are suggestive of methyl bending and deformation vibrations of NIPAM.[214] Peaks at 3302 cm$^{-1}$ and 3078 cm$^{-1}$ are indicative of secondary amide NH stretches and an overtone of the N-H bend, respectfully. The absorbance of the initiator carbonyl stretch (1736 cm$^{-1}$) decreases steadily as the polymerization time increases. This is likely due to the
increased surface coverage of the PNIPAM grafted from the initiated membrane surface. Similarly, the amide carbonyl (1645 cm$^{-1}$), amide bend (1535 cm$^{-1}$), methyl bends (1459 cm$^{-1}$ and 1367 cm$^{-1}$), secondary amide NH stretches (3302 cm$^{-1}$ and 3078 cm$^{-1}$) increase in absorbance and the peak become more distinct with increasing polymerization time. This is indicative of increased polymer brush growth from the membrane surface. Interestingly, the BIBB carbonyl stretch does not completely disappear (as expected) after 8 hours of polymerization. It is likely that some unreacted BIBB is left on the membrane surface which is expected for grafting from ATRP. Typically, the brush thickness obscures any residual BIBB on the membrane surface. Therefore, it is possible that the thickness of the polymer brush on the surface is relatively thin compared to the penetration depth of the ATR IR beam and the BIBB can still be detected. However, the IR results indicate a successful modification of the membrane surface and a thin polymer brush is often desirable to avoid significant changes in the membrane flux.

Captive bubble contact angle measurements for the unmodified, BIBB initiated, and PNIPAM modified RC membranes are shown in Figure 5.4. The lower the contact angle the
more hydrophilic the membrane surface and the higher the angle the more hydrophobic the membrane surface. The unmodified RC membrane surface is the most hydrophilic while the initiated membrane is the most hydrophobic. The large number of hydroxyl groups on the unmodified RC membrane lead to more favorable interactions with water molecules while the presence of the more hydrophobic BIBB initiator after initiation results in a more hydrophobic surface membrane. The PNIPAM modified membrane results in a surface hydrophobicity intermediate to the unmodified and BIBB initiated surfaces. After polymerization the hydrophobicity of the surface due to BIBB is reduced as PNIPAM is grafted from the initiated surface. These results indicate successful initiation and polymer modification of the membrane surface.

Literature reports have shown that PNIPAM modified membrane surfaces demonstrate a change in flux above and below the LCST. Unmodified, BIBB initiated, and PNIPAM modified membranes were characterized by water flux measurements in this work (data not shown). Water flux results do not show definitive thermo-responsive behavior of the PNIPAM brushes, but do show large variations in the absolute water flux between different pieces of
unmodified and PNIPAM modified membranes. The results of these flux measurements showed large variations based on the individual membrane pieces tested. Difference in the membrane properties can exist between RC membranes even on membranes produced by the same method in the same production batch. This means that the membrane properties can vary widely over a relatively small area (several centimeters) of the membrane surface. It is also possible that the polymer brushes exist as a relatively thin layer and that the porosity is not uniform between RC membrane samples. If only a thin polymer layer is present the conformation change at the LCST may not be enough to significantly change the membrane pore size.

5.3.2 Large-scale Membrane: Reactor Design

A major roadblock for bringing covalently modified membranes to real-world applications is the ability to scale up the reaction to modify larger membrane. Often covalent membrane modifications are done on small pieces of membrane (<4 cm$^2$) that fit into a reaction flask.[212–214, 217, 218] However, many applications, including AF4, require large flat sheet membranes that maintain their flatness and porosity. This is a challenge because specialty equipment and large volumes of reagents and reactants are required greatly increasing costs and the integrity of the membrane surface and structure can be compromised. One of the primary goals of this work was to scale up the reaction to enable the modification of large membrane areas (~90 cm$^2$) for use in AF4, a rapidly growing protein, polymer, and nanoparticle characterization technique.[23, 146]

The flatness of the membranes used in AF4 is critical for ensuring separation performance and resolution.[31, 95] This is a significant challenge because the membrane cannot be rolled tightly or folded in efforts to scale up. Both the challenge of maintaining membrane flatness and reducing reagent volumes are overcome by the use of a channel reactor design (Figure 5.5). The channel consists of two plexi-glass blocks that sandwich a thin Teflon spacer and the flat sheet membrane between two Teflon blocks to create a thin channel similar to that of the AF4 channel. Once the channel reactor is assembled a peristaltic pump is used
continually pump the polymerization solution mixture from a three neck round-bottom flask through the channel. This design allows the large-scale membrane to be modified using a reaction mixture of 20 mL in an air-free environment while maintaining a flat membrane surface. Additionally, the materials needed to build the channel reactor, Plexiglas, Teflon sheets, and Teflon tubing, are relatively inexpensive compared to custom glassware large enough to accommodate a large piece of membrane (∼4x40 cm).

Figure 5.5: Schematic of the reactor channel design.

5.3.3 Large-scale Membrane: Characterization

Figure 5.6 shows a comparison of small-scale and large-scale modified membranes. In both the small and large-scale modifications the characteristic bands for amide functional groups (3302 cm⁻¹, 1645 cm⁻¹, 1535 cm⁻¹) are present. Both membranes also show the disappearance of the RC membrane hydroxyl band at 3310 cm⁻¹ and the initiator carbonyl
stretch at 1736 cm\(^{-1}\) indicating that PNIPAM was grafted from the surface. The large scale modification shows slightly less intense absorbance than the small scale membrane from 1400 cm\(^{-1}\) to 1700 cm\(^{-1}\), but the FTIR still suggests successful large scale modification.

Figure 5.6: ATR-FTIR spectra comparison of the small scale and large-scale membrane modifications.

The modification of the large-scale membrane relies on the reaction mixture being pumped through the thin channel to ensure proper mixing. Understanding the extent of the modification along the membrane is important for evaluating how well the method works. Furthermore, even modification over the entire membrane is desirable so that the membrane permeability remains relatively constant throughout the channel and so the membrane surface hydrophilicity remains uniform. Figure 5.7 shows ATR-FTIR along the length of the large-scale modified membrane. The peaks in ATR-FTIR spectra are similar along the membrane suggesting similar amounts of modification (Figure 5.7a). A semi-quantitative analysis of the amide carbonyl peak (1646 cm\(^{-1}\)) allows further evaluation of the channel modification method (Figure 5.7b). There is no significant difference in the max absorbance at 1646 cm\(^{-1}\) along the channel length suggesting that the channel reactor method provides relatively constant PNIPAM modification along the membrane.
PNIPAM is a thermo-responsive polymer that is in an extended, primarily hydrophilic conformation below \( \sim 32^\circ C \) in aqueous solutions. Above \( \sim 32^\circ C \) PNIPAM becomes more hydrophobic and is in a collapsed conformation. This thermo-responsive behavior has interesting applications for changing the membrane properties, but also provides a probe for characterizing and proving successful surface modification. Table 5.1 gives the captive bubble contact angle measurements along the PNIPAM modified membrane length.

Table 5.1: Contact Angle Measurements Above and Below the LCST.

<table>
<thead>
<tr>
<th>Membrane Position (cm)</th>
<th>Contact Angle at 20°C</th>
<th>Contact Angle at 40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>42.5 ± 0.1</td>
<td>45.5 ± 1.0</td>
</tr>
<tr>
<td>6</td>
<td>41.1 ± 0.3</td>
<td>43.2 ± 0.2</td>
</tr>
<tr>
<td>39</td>
<td>43.4 ± 1.1</td>
<td>43.1 ± 0.8</td>
</tr>
<tr>
<td>42</td>
<td>43.3 ± 0.7</td>
<td>46.7 ± 0.3</td>
</tr>
</tbody>
</table>

The thermo-responsive properties of the modified membrane were examined at 20°C and 40°C, below and above the LCST, respectively. The PNIPAM modified membrane has a much higher contact angle than the unmodified membrane at 22°C, but remains relatively...
hydrophilic. However, when the temperature is increased to 40°C a 7% increase in the contact angle is observed. An increase in the captive bubble contact angle indicates that the membrane surface becomes more hydrophobic. Previous work grafting PNIPAM from silicon wafers showed that graft density and polymer molecular weight must be above 0.09/nm² and 48 kDa, respectively, to undergo a thermo-responsive conformation change.[219] Comparison to this work suggests that the brushes on the PNIPAM modified membrane are relatively dense and of a high enough molecular weight to undergo conformation change.[220, 221]

5.3.4 Asymmetrical Flow-Field Flow Fractionation

Membranes were tested in an AF4 channel to assess the performance of the PNIPAM modified membranes. Previous work has used FlFFF as analytical technique to quantify the impact of solution conditions and cross-flow through the membrane to assess reversible and irreversible membrane fouling.[158] In this work, we extend this methodology to examine changes in membrane fouling properties and thermal-responsive behavior.

AF4 retention theory is based on the diffusion of analytes (D) and their average distance from the membrane (l). The retention level (RL) is given by equation 5.1

\[ R_L = \frac{t_r}{t_0} = \frac{w}{6l} = \frac{\dot{V}_c w^2}{6V^0 D} \]  

(5.1)

where \( t_r \) is retention time, \( t_0 \) is the void time, \( w \) is the channel thickness, \( \dot{V}_c \) is the cross-flow rate, and \( V^0 \) is the void volume. The mean layer thickness (l) is the average distance away from the membrane that analytes are positioned during the separation. Figure 5.8 shows fractograms of IVIg using a PNIPAM modified membrane. The IVIg sample was fractionated at 22°C and 37°C to examine the thermo-responsive behavior and hydrophilic/hydrophobic properties of the membrane. However, diffusion is temperature dependent and the diffusion of IVIg and BSA increase with increased temperature resulting in significantly shorter \( t_r \)s if the \( \dot{V}_c/\dot{V}_{out} \) is held constant. This decrease in \( t_r \) is due to the higher \( l \) for analytes at higher temperatures. The \( l \) is a measure of the average distance of the analyte from membrane and hence the smaller the \( l \) the more analyte-membrane interactions occur. Therefore, to
compare the membrane performance at different temperatures the $\dot{V}_e/\dot{V}_{out}$ must be changed so that the $l$ remains constant.

![Fractograms of IVIg (a) and BSA (b) at 22°C and 37°C using the PNIPAM modified membrane.](image)

Figure 5.8: Fractograms of IVIg (a) and BSA (b) at 22°C and 37°C using the PNIPAM modified membrane.

At 22°C the polymer brush is in an extended hydrophilic conformation while above the LCST the brush is in a collapsed more hydrophobic conformation. It has been shown that hydrophobicity plays an important role in protein-membrane interactions with increased membrane hydrophobicity leading to increased protein adsorption. The peak height of the IVIg and BSA peaks decreases as $l$ is decreased for runs at both 22°C and 37°C (Figure 5.8). This behavior is expected because as the $l$ decreases the likelihood of analyte-membrane interactions increases. Furthermore, peak heights for IVIg and BSA decrease as the temperature is increased from 22°C and 37°C while the $l$ is held constant. This indicates increased protein-membrane interactions above the LCST of PNIPAM. The hydrophobicity of the membrane increases above the LCST (~32°C) and increased protein-membrane interactions are expected. Interestingly, at the highest $l$ value (13.5 µm) the peaks have similar heights as the temperature is raised from 22°C and 37°C (although the peaks are still slightly higher at 22°C). This may be due to the faster $tr$ which limits the amount of interactions between the analyte and the membrane during the separation. The ability to characterize protein
adsorption to the membrane surface also demonstrates the potential to use AF4 as method to observe and quantify protein fouling on membranes.

Ideally, the modification of the RC membrane surface with PNIPAM brushes would impart thermo-responsive hydrophilicity changes as well as improve overall sample recovery. The thermo-responsive properties of the modified membrane have been demonstrated by contact angle and AF4 measurements. The sample recoveries for IVIg and BSA were less than 60% and 40%, respectively. These sample recoveries may be due to the moderate molecular weight (MW) and grafting density of the PNIPAM. These PNIPAM MWs and/or grafting densities would allow relatively hydrophobic BIBB initiated surfaces to be exposed to the protein sample which would increase protein adsorption to the membrane. Some membrane interactions are not unexpected as several other publications investigating biocompatible surface modifications have shown that protein adsorption to PNIPAM modified surfaces is possible despite its anti-fouling properties.[219, 222-224] However, the successful surface initiated PNIPAM polymer brushes shown in this work highlights the viability of using a channel reactor for large membrane surface modifications.

5.4 Conclusion

A novel channel reactor was designed to enable the modification of large surface area membranes (∼90 cm²) for subsequent use in AF4 separations of proteins. Until now, only small pieces of membrane (∼4 cm²) that fit into a reaction flask could be covalently modified with polymer brushes. This work highlights the feasibility of the reactor by successfully grafting PNIPAM brushes from the surface of a RC membrane. Characterization of the modified membrane showed that this method provided a uniform modification over the entire membrane surface. Above the LCST of PNIPAM an increase of protein adsorption was observed indicating an increase in hydrophobicity of the membrane surface. This change in hydrophobicity further indicates a successful modification of the membrane and the potential for sample-specific tuning of the membrane surface properties to optimize sample recoveries. The lower than expected protein recovery after modification of the membrane surface and
may be due to the moderate $MW$s and grafting densities of the PNIPAM brushes. The current study demonstrates the ability to successfully scale up the polymerization to enable surface modification of large membrane areas for the first time while reducing the amount of reagents needed.
CHAPTER 6
SEMI-PREPARATIVE ASYMMETRICAL FLOW FIELD-FLOW FRACTIONATION: A CLOSER LOOK AT CHANNEL DIMENSIONS AND SEPARATION PERFORMANCE

The design and optimization of semi-preparative asymmetrical flow field-flow fractionation is investigated to increase sample loading and improve separation performance.

6.1 Introduction

The ability to characterize nanoparticles (NPs) by asymmetrical flow field-flow fractionation (AF4) has impacted a broad range of fields from biotechnology to nanotechnology.[31] Fractionation and purification of analytes is often critical for understanding their fundamental properties and function, and for further characterization and uses.[225] Many analytical scale purification techniques are optimized for relatively small analyte quantities (<100 µg). However, larger quantities (>0.1mg) are often needed for additional analyses and to test the purified samples function in real-world applications. While methods exist for preparative separations of small macromolecules and nano-clusters (<2 nm) [226–228], few exist for NPs.

Precipitation and ultracentrifugation (UC) are often used individually or in combination to obtain NPs that are more narrowly dispersed in characteristics such as size, composition, or morphology.[229] Precipitation allows NPs with different compositions and thus different solubilities to be removed from solution so that they can be characterized or re-suspended in a different solvent. However, precipitation can induce NP aggregation that changes the samples size distribution and potentially alters the properties/behavior of the sample.[230] Furthermore, it can be challenging to re-suspend the sample after precipitation. UC isolates NPs based on their differences in mass.[231] In contrast to precipitation, variation of the centrifugal field and control over the solvent density allows the sample to be isolated into multiple size-density fractions.[232] However, these UC methods must be optimized for each
analyte of interest and often require multiple centrifugation steps that can take hours or days. This places significant limitations on the throughput of UC. Additionally, fraction collection is challenging and sample mixing after the centrifugation is stopped limits the purity of the collected fractions. UC has also been shown to cause aggregation and/or degradation of more delicate biological samples due to the necessary use of high salt concentrations and centrifugal fields. It should be noted that continuous SPLITT fractionation (SF) methods also allow the separation of large sample amounts. Gravitational SF is often used for micrometer or larger sized analytes for example as a method for cell sorting. Centrifugal SF can also be used for continuous sorting of smaller particles and colloids (0.03-3 µm). These promising SF techniques can continuously separate large sample volumes/quantities, but are limited to splitting samples into two distinct populations (small particles and large particles) unless multi-step processes are implemented. Other preparative methods are still required for the separation and collection of more than two size populations with more narrowly dispersed sizes.

Asymmetrical flow field-flow fractionation is a size based separation technique for polymers, proteins, macromolecular complexes, and nanomaterials. Typical analytical scale channels are 100 to 500 µm thick, 25-30 cm long and 2-2.5 cm in breadth. In AF4, the cross-flow field moves the analytes towards the accumulation wall and analyte diffusion causes transport away from the wall. At equilibrium, an exponentially decreasing concentration gradient is formed in the channel with the highest concentration near the semi-permeable membrane accumulation wall. The mean layer thickness (l) represents the center of mass of the analyte cloud and determines retention. The retention parameter (λ) is related to l, the analyte retention time (t_r), the void time (t_0), and the retention level (R_L) by Equation 6.1.

\[ R_L = \frac{t_r}{t_0} = \frac{w}{6l} \]  

Typically, relatively small analyte quantities (1-100 µg) are used for analytical scale AF4 characterization so as to not overload the channel. Excessively large sample quan-
tities introduced into the AF4 channel causes a larger the l for the sample. The retention parameter ($\lambda$) is related to $l$ and the analyte $t_r$. An increase in $l$ will result in a decrease in $t_r$ and leads to inaccuracies in AF4 size characterization when Eq.6.1 is used. Additionally, overloading can cause increased band spreading resulting in decreased separation resolution. Semi-preparative AF4 (sP-AF4)[241, 242] and related techniques [48, 243, 244] have been used to maintain separation performance while increasing sample loading. A creative semi-preparative circular AF4 design used twelve individual channels in a quasi-parallel arrangement to allow the separation of larger sample loads.[243] Hollow fiber flow field-flow fractionation (HF5) utilizes a hollow membrane tube instead of the planar membrane sheet employed in AF4.[244] A multiplexed HF5 system, composed of a bundle of six hollow fiber membranes operated in parallel, was developed to handle sample loads up to 50 $\mu$g.[48] However, the fractionated sample amounts are still relatively low and the use of fibers in parallel is limited due to variations in the properties of each individual fiber, e.g., porosity, flux, surface chemistry.[44]

To overcome the challenges associated with using individual channels or hollow fibers, a large volume AF4 channel has been recently used to separate larger sample quantities. These commercially available channels have a larger channel thickness ($w$) or breadth ($b$). Channels with breadths of $\sim 4.5$ have been used for the separation of magnetic NPs[242] and liposomes[245]. In one study of liposomal drug carriers, sample loads up to 2 mg were successfully separated while maintaining resolution and peak shape.[241] These previous studies demonstrated the use of sP-AF4, but did not examine how channel breadth, shape, and sample type impact sP-AF4 and the limits of sample loading for the commercial channels. Further improvements in separation capabilities, including increasing sample loading, require a more detailed understanding of channel dimensions and their impacts on separation performance. In this work, the design and separation performance of several sP-AF4 channels are examined and the impacts of channel breadth, focusing time, and focusing position are systematically studied. Additionally, the application of organic sP-AF4 separations of
6.2 Materials and Methods

Polystyrene latex (PSL) bead standards of 46 nm and 100 nm (1% solids) were purchased from Thermo Scientific (Waltham, MA). Aqueous suspensions of narrowly dispersed 50 nm and 100 nm silica NPs (10 mg/mL) were purchased from NanoComposix (San Diego, CA). The surfactant mixture FL-70, monosodium phosphate (\( \text{NaH}_2\text{PO}_4 \)), and disodium phosphate (\( \text{Na}_2\text{HPO}_4 \)) were purchased from Fisher Scientific (Fairlawn, NJ). Sodium azide (\( \text{NaN}_3 \)) and tetrahydrofuran were purchased from Sigma Aldrich (St. Louis, MO). Carrier fluid for the PSL beads and silica NPs was composed of 0.03% (v/v) FL-70 and 0.02% (w/v) \( \text{NaN}_3 \) in 1 L of 18.2 MΩ deionized water (AquaMax Ultra, NJ). Phosphate buffer was prepared using 2.670 g \( \text{NaH}_2\text{PO}_4 \) and 4.352 g \( \text{Na}_2\text{HPO}_4 \) in 1 L of 18.2 MΩ deionized water. Tetrahydrofuran was the carrier fluid for the organic AF4 separations. All sample dilutions were done using carrier fluid.

6.2.1 Channel Spacer Dimensions

A variety of channel spacers were created to test the performance of the sP-AF4 systems. All spacers were made of 350 \( \mu \text{m} \) thick Teflon sheets McMaster-Carr (Elmhurst, IL). Figure 6.1 shows the dimensions of different spacers tested in this work.

6.2.2 Aqueous Semi-Preparative Asymmetrical Flow Field-Flow Fractionation

The aqueous AF4 system used a semi-preparative channel (Postnova, Salt Lake City, UT) connected to two LC-6A pumps (Shimadzu, Japan) and a Spectra 100 UV/Vis detector (Spectra-Physics, Santa Clara, CA). The cross-flow rate was controlled using a needle valve. Homemade semi-preparative channel spacers were prepared from 350 \( \mu \text{m} \) thick Teflon sheets. A 30 kDa molecular weight cutoff regenerated cellulose membrane (Microdyn-Nadir, Germany) formed the semi-permeable accumulation wall. A focusing time of 20 minutes was used for PSL standards and silica NP separations unless otherwise specified.
6.2.3 Organic Semi-Preparative Asymmetrical Flow Field-Flow Fractionation

The organic sP-AF4 channel (Postnova, Salt Lake City, UT) was connected to an LC-600 pump (Shimadzu, Japan), a LC-200 pump (Perkin Elmer, Waltham, MA), and a LC-295 UV/Vis detector (Perkin Elmer, Waltham, MA). The 10 cm breadth and 350 µm thick channel spacer was used for all separations. A 10 kDa molecular weight cut-off organic compatible regenerated cellulose membrane (Postnova, Salt Lake City, UT) was employed as the accumulation wall. A 50 kDa polystyrene standard (PSS Polymer Standards, Germany) was used to determine the actual channel thickness. A focusing time of 10 minutes was used for organic AF4 separations.

6.3 Theory of Channel Dimensions

The dimensions of the FFF channel play an important role in the separation performance of a given system. The channel is formed by cutting the desired shape out of a thin (~75 to
500 μm) sheet of Mylar or Teflon. The two most commonly used channel designs are shown in Figure 6.2a and Figure 6.2b. The original flow FFF channels employed a symmetrical channel shape where the breadth \((b)\) was constant along the entire channel length \((L)\) (Figure 6.2a). The cross-flow field is introduced independently of the channel flow through the top wall and passed through the semipermeable membrane covering the accumulation wall allowing for a constant flow velocity throughout the channel.

A second configuration of flow FFF was introduced where a solid block replaced the permeable top wall. In this configuration the fluid entering the channel is split between the channel flow and the cross-flow. However, the use of a rectangular channel in this configuration resulted in a decreased channel flow velocity along the channel length as carrier fluid is continuously depleted through the accumulation wall. This resulted in excessively long retention of analytes and the subsequent introduction of a trapezoidal channel shape where the initial breadth \((b_0)\) at \(z = 0\) decreases to the final breadth \((b_L)\) at \(z = L\) creating an asymmetrical channel to reduce the depletion of cross-flow (Figure 6.2b).

(Figure 6.2c) shows the mean channel flow velocity \((\langle v \rangle_z)\) the at some position \(z\) to along the channel length normalized to the time-averaged mean channel flow velocity \((\langle \bar{v} \rangle)\) versus \(z/L\). The normalized channel flow velocity \((\langle v \rangle_z/\langle \bar{v} \rangle)\) is calculated from Equation 6.2[178]

\[
\frac{\langle v \rangle_z}{\langle \bar{v} \rangle} = \ln \frac{\dot{V}_0}{\dot{V}_L} - \frac{z}{L} + \frac{(1-b_L/B_0)z^2}{2L^2} \left[ 1 - \left(1 - \frac{b_L}{b_0}\right)\frac{z}{L} \right]^{-1}
\]

where \(\dot{V}_0\) and \(\dot{V}_L\) are the channel flow rates at \(z = 0\) and \(z = L\), respectively. The variation of \((\langle v \rangle_z/\langle \bar{v} \rangle)\) with increasing \(z/L\) illustrates how depletion of the cross-flow along the channel impacts the flow velocities in the channel. Additionally, the variation in the \((\langle v \rangle_z/\langle \bar{v} \rangle)\) is dependent on the \(b_L/b_0\) ratio. It has been previously shown that a \(b_L/b_0\) ratio of 0.2 provides the most constant channel flow velocity in trapezoidal channels and that channels where \(b\) decreased exponentially were able to provide uniform channel flow velocities.[178] However, only a specific set of flow rates will provide the constant channel flow velocity and the improvements in separation performance were minor.[246] Therefore, all the semi-preparative
Figure 6.2: (a) Dimensions of a rectangular (symmetrical) channel. (b) Dimensions of a trapezoidal (asymmetrical) channel. (c) Plots of the normalized channel velocity ($\langle v \rangle_z / \langle \bar{v} \rangle$) along $L$ for channels with different $b_L/b_0$ ratios.
channels designed in this work use a trapezoidal geometry with a $b_L/b_0$ ratio of 0.2. The channel aspect ratio ($b/w$) is also important to consider due to frictional drag that occurs at the edge of the channel, termed edge effects. Previous work has evaluated the edge effects in FFF channels and proposed a correction factor based on the channel dimensions.[247] Increasing the aspect ratio by using wider breadth channels was shown to reduce the deviations induced by the edge effects. More recent work suggests that retention is not significantly impacted by frictional drag at the channel edges, even for narrow breadth channels.[248] In either case, increasing the channel breadth reduces any potential frictional drag edge effects that may impact separation performance.

6.4 Results and Discussion

The optimization of sP-AF4 separations including channel breadth, focusing position, and sample loading is discussed below.

6.4.1 Effect of Channel Breadth on Separation Performance

![Fractograms of 46 and 100 nm PSL standards using 2.5, 5, and 10 cm breadth channels. The $\dot{V}_c/\dot{V}_{out}$ was 1.0/0.5, 2.0/1.0, and 4.0/2.0 for the 2.5, 5, and 10 cm channel breadths, respectively.]

Figure 6.3: Fractograms of 46 and 100 nm PSL standards using 2.5, 5, and 10 cm breadth channels. The $\dot{V}_c/\dot{V}_{out}$ was 1.0/0.5, 2.0/1.0, and 4.0/2.0 for the 2.5, 5, and 10 cm channel breadths, respectively.
Figure 6.3 shows the separation of 46 nm and 100 nm polystyrene latex using channels with different b's and a constant $\dot{V}_c/\dot{V}_{out}$ ratio. As the channel breadth increases, the channel and cross-flow velocities decreases if the absolute flow rates are held constant. Changes in these flow velocities will cause differences in the separation performance that are unrelated to b. The channel and cross-flow velocities were kept constant between each channel evaluated to allow for a direct comparison of separation performance. The large void peak (\~1 minute) and non-uniform peak shapes of both the 46 nm and 100 nm PSL indicate significant sample overloading in the 2.5 cm breadth channel. The fractograms for the 5 cm and 10 cm breadth channels show similar separation resolution, but with more symmetric peak shapes. Differences in the UV detector response are likely due to the different channel flow rates used to maintain a constant flow velocity between channels of different breadths. The improvements in peak shape and resolution as well as the reduced analysis time suggests better separation performance using wider channel breadths.

![Figure 6.4: Size determination using AF4 retention theory (a) and resolution (b) of 46 and 100 nm PSL standards as a function of channel breadth.](image)

Figure 6.4 shows the $d_h$s calculated from AF4 retention theory and the resolution of the 46 nm and 100 nm PSL with increasing b. Accurate sizes for both PSL standards are calculated from their $t_r$s measured using the 5 cm and 10 cm breadth channels (Figure 6.4a). The
sizes determined using the 2.5 cm breadth channel are lower than the nominal $d_h$s and those determined on the 5 cm and 10 cm breadth channels. This is due to the sample overloading observed in the 2.5 cm breadth channel. Overloading is caused by high concentrations of sample at the membrane accumulation wall that inhibits full relaxation of the sample to its equilibrium mean layer thickness ($l$). The high concentration forces sample further away from the wall and results in analytes experiencing faster flow regimes than predicted by AF4 retention theory. This results in early sample elution and erroneously small sizes calculated. Figure 6.4b shows the resolution between the PSL standards at different channel $b$s. Better resolution is observed using the 5 cm and 10 cm breadth channels. The lower resolution for the 2.5 cm breadth channel is again due to overloading of the channel. The similar performance observed for the 5 cm and 10 cm breadth channels shows this expected to handle larger sample amounts than the 5 cm channel and was thus chosen for further studies evaluating sP-AF4 channel performance.

6.4.2 Effect of Focusing Time and Focusing Band Position on Separation Performance

Sample focusing is a unique and important step in AF4. The details of the focusing procedure are different depending on the configuration of the AF4 system, but the procedure always involve 3 main parts:

1) Sample introduction into the channel
2) Equilibration of the sample near the accumulation wall (sample relaxation)
3) Formation of a narrow sample band near the beginning of the channel

The flows entering the channel are set such that sample is introduced into the channel, relaxed near the accumulation wall, and focused into a narrow band. Figure 6.5 shows the effect of increasing focusing time on the separation of 46 nm and 100 nm PSL standards. As expected, increasing the focusing time from 5 minutes to 20 minutes results in a decrease in the void peak and an increase in the retained peaks. This suggest that a sample focusing time of 20 minutes is sufficient for sample relaxation and is used in subsequent studies.
Figure 6.5: Fractograms of 46 and 100 nm PSL standards using focusing times of 5 to 20 minutes. The $\dot{V}_c/\dot{V}_{out}$ was 4.0/2.0 for all runs.

The focusing position is also an important parameter yet its effect has not been well studied. Adjusting the inlet and focusing flow rate ratio allows the focusing position to be moved closer or further from the channel inlet. The pictures in Figure 6.6a show the focusing position and shape of a band of blue dye inside the channel. The blue dye clearly shows that the focused band has a curved shape as the focusing position increases from 2 to 11 cm (at 11 cm the position of the blue dye is partially obscured by the stainless steel block). At 17 cm the blue dye is positioned near the focusing inlet. As fluid enters the channel it spreads radially from the focusing inlet resulting in the curvature observed for the 17 cm focusing position. It is also apparent that blue dye is able to pass the focusing inlet. The curvature observed for the 2 to 11 cm bands is due to the triangular ends utilized in FFF channels and the subsequent different path lengths traveled by sample depicted in Figure 6.6b. The curvature of the sample band in the channel has been previously modeled theoretically, observed experimentally, and the plate height contribution of the curvature was calculated.[249] The
Figure 6.6: (a) Pictures showing the focusing position of blue dye inside the channel. A stainless steel block with circular cut-outs that expose the AF4 channels Plexiglas top wall allows visual examination of the blue dye band. The numbers over each picture correspond to the distance from the channel inlet (located at the top of the picture in all cases). (b) Depiction of the sample band curvature due to the triangular end piece of the FFF channel. The solid black lines trace the FFF channel shape at the inlet and as it progresses towards the outlet. (c) Fractograms of 46 and 100 nm PSL standards using focusing positions at 2 to 17 cm from the channel inlet. The $\dot{V}_c/\dot{V}_{out}$ was 4.0/2.0 for all runs.
plate height contribution of the curvature can be changed by modifying the angle of the triangular end and the influence of the triangular ends on separation performance is the subject of future research.

Figure 6.6c demonstrates the effect of focusing position on the separation of PSL standards. As the focusing position is moved away from the channel inlet, both PSL standards shift towards earlier $t_r$s. This is expected because the effective channel length is decreased. At 11 cm and 17 cm the focusing position approaches the point of the focusing inlet and the resolution and $t_r$s decrease significantly. This is likely due in part to the short effective channel length and the decrease in $b$ further down the channel. At focusing positions of 11 cm and 17 cm, the channel breadth is $\sim$5 cm and 3 cm, respectively. The large void peaks and poor resolution suggest that overloading is occurring at these focusing positions due to the decreased channel breadth. These results suggest focusing as close to the channel inlet provides the highest resolution and that focusing positions less than 25% of the channel length provide a good compromise between resolution and maximum sample loading.

### 6.4.3 Effect of Sample Loading on Resolution

Figure 6.7 shows the effect of increasing sample loading of 50 nm and 100 nm solid silica spheres on the sP-AF4 separation. Sample loads in analytical scale AF4 are typically 1-100 $\mu$g. In this work, sample loads are increased from 400 $\mu$g to 20 mg representing an increase over one order of magnitude. The absorbance of silica particles in the UV detector is small and even at a relatively high sample loading of 400 $\mu$g the UV response is very low. A ten times increase in the sample load to 4 mg results in a much higher UV response and the peaks have a resolution of $\sim$1.3 (Figure 6.7b). A further increase to 10 mg sample load yielded peaks that are slightly less resolved, but with no significant sign of overloading. For a sample load of 20 mg, the resolution decreases to 0.8 which is still sufficient for producing relatively pure NP size fractions. This sample loading study demonstrates the capabilities of sP-AF4. The ability to successfully separate several tens of mg of sample in under 30 minutes is important for improving throughput and obtaining sufficient quantities for subsequent NP
6.4.4 Organic Semi-Preparative Asymmetrical Flow Field-Flow Fractionation

Hybrid nanoparticles (HNs) are composed of multiple particle domains connected by solid-state interfaces. The combination of distinct inorganic domains result in particles that exhibit multiple functionalities and synergistic properties.[250–256] However, the synthesis of HNs with narrow size and morphological distributions is challenging.[229, 257] Syntheses require multiple steps where the size of each domain must be controlled. Furthermore, multiple inorganic domains can be added during each synthesis step resulting in even more sample polydispersity. In this work, the size polydispersity of HNs composed of Pt and Fe$_3$O$_4$ domains were analyzed by organic sP-AF4. The synthesis begins with the growth of Pt nanocubes that are ~6-7 nm across on each face. Then Fe$_3$O$_4$ lobes are then grown from the Pt nanocube seed particles. Nucleation tends to occur at the corners of the Pt nanocubes resulting in Pt-Fe$_3$O$_4$ nanoflowers with one to potentially eight Fe$_3$O$_4$ lobes attached. Addition of each Fe$_3$O$_4$ domain can potentially change the properties of the HNs, but the wide size and morphological polydispersity of these samples makes it difficult to
determine their structure-function relationship.

Figure 6.8: (a) Fractograms of $Fe_3O_4$ lobes and $Pt-Fe_3O_4$ nanoflowers. (b) Fractograms of $Pt-Fe_3O_4$ with the corresponding $r_{rms}$ determined by MALS. (c) Fractograms of $Pt-Fe_3O_4$ nanoflowers with increasing sample amounts. The $\dot{V}_c/\dot{V}_{out}$ was 2.0/0.25 and a focusing time was 6 minutes for all runs.

Organic sP-AF4 has not been previously demonstrated in published literature. The HNs used in this work are stable in organic solvents that are incompatible with common aqueous AF4 systems. Figure 6.8a shows the separation of the $Pt-Fe_3O_4$ nanoflowers and $Fe_3O_4$ lobes with similar sizes to the lobes attached to the nanoflowers. Each sample injection contains $\sim 100 \ \mu g$ of each particle type. The comparison of the TEM determined diameter and the $d_h$ determined by organic sP-AF4 demonstrate good agreement (Table 6.1). Figure 6.8b
shows the fractograms and $r_{rms}$ for the $Pt-Fe_3O_4$ nanoflowers. The $r_{rms}$ demonstrates the successful size separation of the $Pt-Fe_3O_4$ nanoflowers. Interestingly the $r_{rms}$ has two distinct regions (1) from 7.5 to 20 minutes and (2) from 20 to 35 minutes. The first region has a uniform $r_{rms}$ of $\sim$7.5 nm while the size range of the second region shows an increase in size from 7.5 to 17 nm. These two $r_{rms}$ size ranges correspond closely to the TEM and AF4 sizes determined for the Fe lobes and $Pt-Fe_3O_4$ nanoflowers, respectively (Table 6.1). The two distinct regions suggest that there may be a population of both $Pt-Fe_3O_4$ nanoflowers and a population of free Fe lobes.

Table 6.1: TEM and AF4 determined sizes for Fe$_3$O$_4$ and Pt-Fe$_3$O$_4$.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sample Amount (mg)</th>
<th>TEM Size (nm)</th>
<th>AF4 Size (nm)</th>
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<tr>
<td>Fe$_3$O$_4$</td>
<td>0.10</td>
<td>11</td>
<td>12.1</td>
</tr>
<tr>
<td>Pt-Fe$_3$O$_4$</td>
<td>0.02</td>
<td>27</td>
<td>29.6</td>
</tr>
<tr>
<td>Pt-Fe$_3$O$_4$</td>
<td>0.10</td>
<td>27</td>
<td>32.0</td>
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<tr>
<td>Pt-Fe$_3$O$_4$</td>
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<td>19.4</td>
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<tr>
<td>Pt-Fe$_3$O$_4$</td>
<td>0.60</td>
<td>27</td>
<td>17.7</td>
</tr>
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Figure 6.8c shows the fractionation of increasing amounts of $Pt-Fe_3O_4$ nanoflowers up to $\sim$600 $\mu$g. The shift in retention time from 20 minutes to 12.5 minutes as the sample load increases is indicative of channel overloading. This is most apparent as the sample load is increased from 100 $\mu$g to 200 $\mu$g. The sizes reported in Table 6.1 demonstrate the impact of overloading on size determination by AF4. Below 100 $\mu$g the AF4 sizes are similar to those determined by TEM and MALS. Above the AF4 sizes decrease significantly due to the shift in retention to earlier times. These sample loads 200 $\mu$g indicate that overloading occurs at much lower sample amounts than previously observed for silica nanospheres (Figure 6.7). It is well known that overloading is sample dependent and it is possible that large electrostatic screening lengths present between the $Pt-Fe_3O_4$ NPs cause overloading to occur at much lower sample amounts in organic solvents. However, despite the shift in retention time and changes in AF4 determined size caused by overloading, separation of the samples is still
possible. Therefore, size separated fractions can be collected (even from overloaded analyses) and used for additional composition and morphological analyses.

6.5 Conclusion

The separation and purification of nanomaterials is important for understanding their fundamental properties and large sample quantities are often required for further studies. In this work, sP-AF4 channels to handle milligram sample quantities are designed and their separation performance is investigated. Consideration of the impacts of channel dimensions on separation performance suggests that increasing the channel breadth (to increase the channel volume and allow for larger sample amounts) is a viable design route for sP-AF4 channels. Experimental results confirmed that wider breadth channels had the best resolution for the analytes and channels examined in this work. The position of sample focusing band plays an important role in sample retention and resolution. The optimal focusing position is close to the inlet and a position within 25% of the channel length of the inlet. It should be noted that the effect of focusing band position demonstrated in this work is also applicable to analytical scale AF4 separations. Fractionation of up to 10 mg of 50 and 100 nm silica NPs is demonstrated without significant overloading effects. Higher sample loadings (up to 20 mg) are also possible, but overloading begins to reduce resolution. Finally, organic sP-AF4 is demonstrated for the first time to separate HNs and the sample loading amount is significantly lower than for the silica NPs. The development of both aqueous and organic compatible sP-AF4 channels shows the possibilities for the fractionation of milligram quantities of nanomaterials to allow for orthogonal characterizations and applications.
CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

The developments described in this dissertation have addressed several key limitations currently facing asymmetrical flow field-flow fractionation (AF4). These advances allow for further expansion of AF4 as a primary technique for the separation and characterization of nanoparticles, macromolecules, and proteins.

7.1 Conclusions

In Chapter 3, the development of an AF4-MALS method to separate protein aggregates allowed for the characterization of LENP aggregation kinetics over the submicron size range for the first time. Centrifugation (a common sample preparation method used prior to SEC) was found to remove the largest aggregate species which may alter the SEC determined aggregation kinetics. The misperception of high sample dilution during AF4 analysis was addressed in Chapter 4. Relatively low sample dilution is experienced during AF4 and occurs primarily at the channel outlet, not during fractionation. This suggests that AF4 retention theory provide more accurate size information for rapidly dissociating protein aggregates (and other supramolecular assemblies) than online light scattering detectors. Chapter 5 has addressed perhaps the biggest weaknesses of AF4: analyte-membrane interactions. The successful development of a method to graft PNIPAM polymer brushes from large membrane surfaces provides the groundwork necessary to reduce unwanted protein-membrane interactions. This work not only applies to protein-membrane interactions, but also can be applied to the analysis of polymers and nanoparticles where membrane interactions pose a significant challenge. Finally, the design and optimization of AF4 channels to purify up to 20 mg of sample in aqueous and, for the first time, organic compatible channels represents a significant advance for sP-AF4. This allows for preparation of narrowly dispersed size
populations to understand their fundamental properties by further characterization or for additional applications.

7.2 Future Directions

The number of AF4 publications has expanded rapidly over the past 20 years. However, critical limitations still exist and have hindered more widespread adoption of AF4. The work in this thesis has addressed a number of these limitations, and has also laid the groundwork for a number of future advances. Unwanted analyte interactions with the ultrafiltration membranes is a persistent challenge for AF4. The development of a method to covalently modify the membrane surface with polymer brushes has provided a route to overcome this challenge. The work in this thesis focused on protein-membrane interactions, but similar interactions exist during the analysis of synthetic and environmentally relevant inorganic nanoparticles and biological, natural, and synthetic polymers. The method developed in chapter 5 should be extended to different polymer chemistries and covalent modifications to reduce and control a variety of unwanted interactions. For protein analyses alone a wide range of homo- and co-polymers including polyethylene glycol, polyacrylonitrile, and polymeric betaines should be investigated. Additionally, the use of stimuli-responsive polymers can be explored to create membranes with tunable surface properties that respond to temperature, solution conditions (pH, ionic strength), light, and/or electric fields. These potential applications for AF4 could also be extended to other analytical techniques and industrial processes that use membranes and experience similar issues with membrane fouling.

sP-AF4 has a wide range of potential applications in the future. The ability to separate large sample quantities and collect narrowly size dispersed fractions allows for the expansion of two-dimensional separation and characterization techniques. The work in this thesis can be directly extended to the two-dimensional analysis of HNs. A two-dimensional approach would allow for HN characterization by size and by composition which is critical for understanding the unique properties of these complex nanomaterials. Other biological applications are also now viable with the advances presented in this work. For example, ultracentrifugation is often
used to isolate various proteins and vesicles from human blood plasma samples to enable the discovery and characterization of disease biomarkers. However, centrifugation is time consuming and often requires high salt concentrations (>1 M) which can lead to degradation of materials of interest. sP-AF4 has the potential to augment or replace centrifugation as a preparation method for these size disperse biological samples. Preliminary results have shown that sP-AF4 provides similar sample amounts and higher sample recoveries compared ultracentrifugation preparations of lipoproteins form human plasma samples.
REFERENCES CITED


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Figure A.1 and Figure A.2

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![Figure A.1](https://example.com/figureA1.png)

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Figure A.2

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