1 **Semi-Preparative Asymmetrical Flow Field-Flow Fractionation: A Closer**
2 **Look at Channel Dimensions and Separation Performance**

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Abstract

The design and performance of a semi-preparative asymmetrical flow field-flow fractionation (SP-AF4) channel are investigated with the objective of better understanding and exploiting the relationship between channel dimensions, sample loading, and resolution. Most size-based separations of nanometer and submicrometer particles are currently limited to analytical scale quantities ($<100\text{ }\mu\text{g}$). However, there is a strong need to fractionate and collect larger quantities so that fundamental properties of the more narrowly dispersed fractions can be studied using additional characterization methods and for subsequent applications. In this work, dimensions of the spacer that defines the form of SP-AF4 channels are varied and their performances are assessed with respect to sample focusing position and loading. Separations are performed in aqueous and organic carrier fluids. A critical evaluation of channel dimensions showed that increasing the channel breadth is a practical and effective route to maintaining separation resolution while increasing sample loads to milligram quantities. Good size resolution (~ 1.0) is achieved for separations of 10 mg of 50 and 100 nm silica nanoparticles suspended in water and up to 0.6 mg of ~ 10 to 35 nm inorganic hybrid nanoparticles suspended in tetrahydrofuran. This work represents important advances in the understanding of SP-AF4 separations and extends sample loading capacities in both aqueous and organic solvents.

1. Introduction

The separation and characterization of nanoparticles (NPs) by asymmetrical flow field-flow fractionation (AF4) has impacted a broad range of fields from biotechnology to nanotechnology.[1] Synthesized nanomaterials can be polydisperse in various properties and the availability of fractions with narrower distributions is often critical for additional characterization and applications-related studies that more definitively correlate their fundamental properties and observed functions.[2] Such studies are limited by the $< \sim 100 \mu\text{g}$ amounts of sample produced by analytical scale separation techniques. While methods exist for preparative separations of macromolecules and nano-clusters with sizes below $\sim 2 \text{ nm}$ [3–5], few exist for NPs larger than $\sim 2 \text{ nm}$.

Precipitation and ultracentrifugation (UC) are often used individually or in combination to obtain NPs that are more narrowly dispersed in size, composition, or morphology.[6] For example, gold NPs with a specific size and shape can be precipitated from aqueous salt solutions.[7] Precipitation conditions are often specific to the analyte composition, involve multiple steps, and require the addition of select solvents and salts that are not easily applied to other NP types and suspensions.[8,9] Precipitation can also induce irreversible NP aggregation that changes the sample's size distribution and potentially alters the properties and behavior of the sample.[10] Furthermore, it can be challenging to re-suspend the sample after precipitation. Ultracentrifugation isolates NPs based on their differences in mass.[11] In contrast to most precipitation methods, variation of the centrifugal field and selection of the solvent density allows the sample to be separated into a spectrum of different masses.[12] It is important to note that each mass fraction can be composed of different size and density components and thus the extraction of size information requires knowledge of the NP density within each mass fraction.

Furthermore, UC methods often require multiple centrifugation steps that can take hours or days.[13] This places significant limitations on the throughput of UC. Fraction collection is also challenging and sample re-mixing after the centrifugation is stopped limits the purity of the collected fractions. Ultracentrifugation 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.[14] Continuous SPLITT fractionation methods have been demonstrated for the separation of large sample amounts (~15 grams per hour).[15] Gravitational SPLITT is typically used for micrometer-sized analytes such as cells[16–18] while the higher fields employed in centrifugal SPLITT extended the range down to ~0.03 μm particles[15,19,20]. These promising techniques have been used to continuously separate large sample volumes and quantities but are typically limited to splitting samples into two distinct mass or size populations unless time consuming multi-step processes are implemented.[21] There is an unmet need for semi-preparative or preparative scale techniques that can readily produce an array of narrowly dispersed sample fractions in amounts > 100 μg and with minimal alteration of the sample.

Asymmetrical flow field-flow fractionation (AF4) is a size based separation technique with a demonstrated applicability to diverse samples that include polymers, proteins, macromolecular complexes, nanomaterials, and cells.[22–24] AF4 is the most commonly used variation in the FFF family of techniques, partly because of the relatively straightforward data interpretation. Retention of sub-micrometer particles is dependent on diffusion coefficients that in turn are related to hydrodynamic sizes. Hence, the resultant signal versus retention time plot or fractogram is representative of and readily converted to a size distribution.

The AF4 assembly is formed by sandwiching a spacer between a solid block and a semi-permeable block that consists of a frit covered by a semi-permeable membrane. Typically, trapezoidal shapes are cut out of Teflon or Mylar sheets or spacers to form the AF4 channel (Figure 1). The cut out area of the analytical scale spacer or channel has typical dimensions of ~20-30 cm in length (L), 2-2.5 cm in maximum breadth (b_o), 0.5-1.0 cm in minimum breadth (b_L), and 75 to 500 μm in thickness (w). A parabolic channel flow is formed across w moving along the L axis and a perpendicular cross-flow field is formed as fluid leaves through the semi-permeable wall. The cross-flow field also moves analytes towards the semi-permeable accumulation wall and analyte diffusion causes transport away from this wall. At equilibrium, an exponentially decreasing concentration gradient is formed in the channel with the highest concentration near the membrane accumulation wall. The sample is then carried down the channel by the parabolic channel flow with different components transported at different speeds characteristic of their diffusion coefficients and subsequent equilibrium positions in the different velocity streamlines of the parabolic flow profile. The mean layer thickness (l) represents the distance of the center of mass of the analyte cloud from the accumulation wall and determines retention. The retention level (R_L)[25] is related to l , w , the analyte retention time (t_r), and the void time (t^0) by Equation 1.

$$R_L = \frac{w}{6l} = \frac{t_r}{t^0} \quad \text{Eq.1}$$

Sample quantities $<100 \mu\text{g}$ are typically used for analytical scale AF4 [26] so as to not overload the channel.[27] Overloading is a phenomenon where the t_r is shifted and the peak symmetry is skewed due to excessively high sample concentrations near the accumulation wall. This mainly occurs during the focusing/relaxation step prior to AF4 separation where the sample is focused into a narrow band near the start of the channel and allowed to form the steady state

1 concentration distribution. Under overloaded conditions, the too high sample concentration
2 distorts the expected equilibrium concentration distribution. Additionally, overloading can cause
3 increased band spreading resulting in decreased separation resolution.[27]

4 Semi-preparative AF4 (SP-AF4) can be performed using multiple channels
5 simultaneously and collecting the combined effluent or by increasing the dimensions of a single
6 channel.[28–32] For example, a circular SP-AF4 design used twelve individual AF4 channels in
7 a “quasi-parallel arrangement” akin to the petals of a flower. This design allowed for the
8 separation of 2.5 mg of sample with resolutions of ~1.2 and 1.3 between 50 and 100 nm and 100
9 and 200 nm polystyrene latex (PSL) beads, respectively.[30] Hollow fiber flow FFF (HF5) is a
10 variant of FFF that utilizes a membrane tube instead of the planar membrane sheet employed in
11 AF4.[31] A multiplexed HF5 system, composed of a bundle of six hollow fiber membranes
12 operated in parallel, was developed to handle sample loads up to 50 μg .[32] However, the
13 fractionated sample amounts are still relatively low and the use of individual channels or fibers
14 in parallel is limited due to potential variations in the porosity, flux, and surface chemistry of
15 each fiber.[33] Using a single channel designed to handle large sample quantities would help
16 resolve some of these limitations.

17 Semi-preparative AF4 can also be achieved using a single AF4 channel with increased
18 spacer dimensions (L , w , b_0 , and b_L). Large volume AF4 channels (~3 mL) with b_0 dimensions
19 of up to ~4.5 cm have been used to separate magnetic NPs[29] and liposomes[34]. In one study
20 of liposomal drug carriers, sample loads up to 2 mg were successfully separated.[28] These
21 previous studies demonstrated the use of SP-AF4, but did not examine how channel dimensions
22 impact separations or determine the limits of sample loading using different samples or carrier
23 liquids.

Further improvements in separation capabilities, especially increasing sample loads, require a more detailed evaluation of channel dimensions and their impacts on separation performance. Sample overloading is most likely to occur during the relaxation/focusing step where the sample is constrained by the channel w and breadth (at or near b_0). The length L does not have an impact on overloading during the relaxation/focusing step. The spacer w can be increased to produce a larger channel volume capable of handling larger sample amounts. However, nonequilibrium band broadening (H_{neq}), the largest contributor to peak broadening during AF4, scales as the square of w . Thus increasing w would result in a significant increase in broadening.[35] The channel volume can also be increased by increasing b_0 and b_L . Increasing the breadth will reduce the impact of frictional drag at the channel edge on the channel flow velocity [36] and does not contribute to broadening. A more in depth look at the theory of channel dimensions is discussed in the next section.

In this work, the relationships between channel breadth, sample load, and separation resolution in SP-AF4 are systematically studied. Additionally, organic SP-AF4 separations are demonstrated for the first time using a complex sample system of hybrid Pt-Fe₃O₄ nanoparticles.

2. Theory

In AF4, the fluid entering the channel is subsequently divided into the channel flow and the crossflow.[37] However, the use of a rectangular spacer shape ($b_0 = b_L$) results in a decreased channel flow velocity along the channel length as carrier fluid is continuously depleted through the accumulation wall.[38] This can lead to excessively long retention of analytes. To address this issue, a trapezoidal spacer shape where the initial breadth at $z = 0$ (b_0) decreases to the final breadth (b_L) at $z = L$, is used to maintain the channel flow velocity and ensure a reasonable analyte t_r (Figure 1).

The mean channel flow velocity ($\langle v \rangle_z$) at some position z along the trapezoidal AF4 channel length is calculated from Equation 2[39]

$$\langle v \rangle_z = \langle v \rangle_0 \left[1 - \frac{2(1-\dot{V}_L/\dot{V}_0)}{(1+b_L/b_0)} \times \left\{ \frac{z}{L} - \frac{(1-b_L/b_0)}{2} \frac{z^2}{L^2} \right\} \right] \times \left(1 - \left(1 - \frac{b_L}{b_0} \right) \frac{z}{L} \right)^{-1}$$

Eq. 2

where $\dot{V}_0$ and $\dot{V}_L$ are the channel flow rates at $z = 0$ and $z = L$, respectively. The results plotted in Figure 2 show the variation of $\langle v \rangle_z$ along L and illustrate how depletion of the crossflow along the SP-AF4 channel impacts the flow velocities in the channel. Additionally, the variation in $\langle v \rangle_z$ 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 (but not entirely) uniform channel flow velocity in trapezoidal channels. This is important in certain situations where the channel flow velocity becomes too low and could cause undesirable effects like excessively long retention times or zone broadening. Hence, it is important to choose a b_L/b_0 ratio that does not cause a significant decrease in channel flow velocity. Spacers where b_0 decreases exponentially to b_L are able to provide entirely uniform channel flow velocities[39], but a specific set of flow rates must be used and the improvements in zone broadening are not significant.[40] The commercial triangular channel would have a $b_L/b_0 < 1/10$ where the $\langle v \rangle_z$ increases dramatically near the channel outlet (Figure S1). Based on these considerations, the semi-preparative spacers designed in this work use a trapezoidal geometry with a b_L/b_0 ratio of 0.2.

The triangular end pieces of the channel cause the carrier fluid to fan out from the channel inlet resulting in a crescent shaped sample band representative of equal distances from the inlet.[41] Previous work has shown that increasing the triangular end piece angle (at the inlet and outlet) increased the distortions in the flows across the channel breadth and led to incremental increases in band broadening.[41] However, the broadening caused by the triangular

end pieces is likely to be significantly lower (~ 1 order of magnitude) than broadening due to nonequilibrium effects. Therefore, the influence of the triangular end pieces on the separation performance of the SP-AF4 channel is only briefly discussed in this work.

The spacer aspect ratio (b/w) should also be considered due to frictional drag that occurs at the edge of the channel, termed edge effects. Edge effects in FFF channels have been evaluated and a correction factor was proposed based on the channel dimensions.[36] For spacers with aspect ratios >40 (most analytical and semi-preparative spacers), edge effects are negligible ($< 1\%$ change in channel flow velocity). Furthermore, recent work suggests that retention is not significantly influenced by edge effects.[42] Hence, edge effects are not expected to significantly influence retention or resolution in SP-AF4 and are therefore not investigated in this work.

3. Methods and Materials

3.1 Materials

Polystyrene latex (PSL) bead standards with diameters of 46 nm and 100 nm (1% solids) are purchased from Thermo Scientific (Waltham, MA, USA). Aqueous suspensions of narrowly dispersed 50 nm and 100 nm silica NPs (10 mg/mL) were from NanoComposix (San Diego, CA, USA). Oleylamine (OLAM; technical grade, 70%), sodium azide (NaN_3) and tetrahydrofuran (HPLC grade) are purchased from Sigma Aldrich (St. Louis, MO, USA). The surfactant mixture FL-70, monosodium phosphate (NaH_2PO_4), and disodium phosphate (Na_2HPO_4) were from Fisher Scientific (Fairlawn, NJ, USA). Carrier fluid for the PSL beads and silica NPs is composed of 0.03% (v/v) FL-70 and 0.02% (w/v) NaN_3 in 1 L of 18.2 M Ω deionized water (AquaMax Ultra, NJ, USA). Phosphate buffer is prepared using 2.670 g NaH_2PO_4 and 4.352 g Na_2HPO_4 in 1 L of deionized water. Tetrahydrofuran was the carrier fluid for the organic AF4 separations. All sample dilutions are done using carrier fluid.

3.2 Synthesis and Characterization of Pt-Fe₃O₄ Hybrid Nanoparticles

Iron oxide (Fe₃O₄) and Pt-Fe₃O₄ hybrid nanoparticles were synthesized following previously reported procedures.[43] The final product morphology yielded Pt cubes with multiple Fe₃O₄ domains. A 1200 EX II transmission electron microscope (JEOL, Peabody, MA, USA) at an accelerating voltage of 80 kV was used to collect images and determine sizes of the synthesized particles. Size information was also obtained from SP-AF4 retention times.

3.3 Aqueous SP-AF4 System

The aqueous AF4 system used the housing (top block, bottom block, and stainless steel frit) of a semi-preparative channel (Postnova, Salt Lake City, UT, USA), two LC-6A pumps (Shimadzu, Japan), and a Spectra 100 UV/Vis detector (Spectra-Physics, Santa Clara, CA, USA). The crossflow rate was controlled using a needle valve. The Postnova spacer had a triangular inlet with a maximum b_0 of 10 cm and a triangular channel with a length of 24 cm (Figure S1) rather than the commonly used trapezoidal shaped channel. Therefore, home-made trapezoidal spacers were created to test the performance of the SP-AF4 system. All spacers were made of 350 μ m thick Teflon sheets (McMaster-Carr, Elmhurst, IL, USA). The focusing flow inlet was position 18 cm from the channel inlet. Complete homemade spacer dimensions are given in Table 1. A 30 kDa molecular weight cutoff regenerated cellulose membrane (Microdyn-Nadir, Germany) formed the semi-permeable accumulation wall. The 46 and 100 nm PSL standards are used to determine the actual w_s of each channel. A focusing time of 20 minutes was used for PSL standards and silica NP separations unless otherwise specified.

3.4 Organic SP-AF4 System

The organic compatible SP-AF4 channel housing (Postnova, Salt Lake City, UT, USA) with home-made spacers was connected to an LC-600 pump (Shimadzu, Japan), a LC-200 pump (Perkin Elmer, Waltham, MA, USA), and a LC-295 UV/Vis detector (Perkin Elmer, Waltham, MA, USA). A DAWN EOS multiangle light scattering detector (Wyatt Technologies, Santa Barbara, CA, USA) was used to determine the root mean square radius (r_{rms}) distribution of the Pt-Fe₃O₄ NPs. The spacer with a b_0 of 10 cm breadth and a w of 350 μ m was used for all separations. A 10 kDa molecular weight cut-off organic compatible regenerated cellulose membrane (Postnova, Salt Lake City, UT, USA) was employed as the accumulation wall. A 50 kDa polystyrene standard (PSS Polymer Standards, Germany) was used to determine the channel thickness. A focusing time of 10 minutes was used for organic SP-AF4 separations of Fe₃O₄ and Pt-Fe₃O₄ NPs.

4. Results and Discussion

4.1 Effect of Channel Breadth on Separation Performance

Figure 3 shows the separation of 46 nm and 100 nm PSL beads using spacers with different breadths, constant b_L/b_0 ratios of 0.2, and constant $\dot{V}_c/\dot{V}_{out}$ ratios of 2. If the absolute flow rates are held constant as the spacer breadth increases, the channel and cross flow velocities will decrease and differences in the separation performance will be due to changes in flow velocities rather than to changes in breadth. Therefore, the channel and cross flow rates were selected such that the same flow velocities are maintained for all spacer breadths, e.g., $\dot{V}_c/\dot{V}_{out}$ of 0.5/1.0, 1.0/2.0, and 2.0/4.0 for b_L/b_0 of 0.5/2.5, 1.0/5.0, and 2.0/10 cm, respectively. The use of the same flow velocities and $\dot{V}_c/\dot{V}_{out}$ of 0.5 is expected to result in identical t_r s for the PSL beads regardless of the channel breadth based on AF4 retention theory. It should be noted that the 0.5/2.5 spacer has dimensions typical of analytical scale systems so that comparisons can be

made between analytical and semi-preparative channels. Figure 3 shows that the t_r for both the 46 nm and 100 nm PSL unexpectedly changes (decreases) with increasing channel breadth. This is due to differences in channel thicknesses. A nominal w of 350 μm was assumed during the selection of flow rates to maintain a constant flow velocity. Experimentally calculated w values of 294, 279, and 247 μm for the 0.5/2.5, 1.0/5.0, and 2.0/10.0 cm spacers (or channel) breadths, respectively, are based on the nominal size of 46 nm and 100 nm PSL beads standards. The determination of w has a $\sim 5\%$ error for a well-retained analyte ($R_L \geq 5$). [25] Since the membrane was not replaced with each spacer change, it is possible that the membrane became more compressed due to the application of crossflow over time (leading to larger w values). The 2.0/10.0 cm spacer was used first, the 1.0/5.0 cm spacer was used second, and the 0.5/2.5 cm spacer was used third. Therefore, difference in w between channels is likely due to the age of the membrane. Lower w values would result in higher than expected channel flow velocities resulting in shorter a t_r and is likely the cause for the differences in t_r observed between spacers of different breadths. The large void peak at ~ 1 minute and asymmetric peak shapes of both the 46 nm and 100 nm PSL using the 0.5/2.5 cm spacer indicate possible sample overloading. The fractograms for the 1.0/5.0 and 2.0/10.0 b_L/b_0 spacers show smaller void peaks, more symmetric peak shapes, and no evidence of overloading. Differences in the UV detector response are likely due to the different channel flow rates used to maintain a constant flow velocity between spacers of different breadths. Correcting for the difference in flow rates between the 1.0/5.0 cm and 2.0/10.0 cm (b_L/b_0) channels yield similar peak areas of 1.7 V $\cdot$ mL and 1.9 V $\cdot$ mL, respectively. The improvements in peak shape and smaller void peaks for the 1.0/5.0 and 2.0/10 cm spacers suggest better separation performance for large analyte quantities using wider spacer breadths compared to analytical scale dimensions.

Figure 4 shows d_h values calculated from AF4 retention theory and the resolution of the 46 nm and 100 nm PSL with increasing channel breadths. The sizes for both PSL standards are calculated from their t_r for all spacer breadths (Figure 4a) and are in agreement with nominal values. The resolution between the 46 nm and 100 nm PSL beads is significantly lower using the 0.5/2.5 cm breadth spacer compared to the 1.0/5.0 cm and 2.0/10 cm spacers (Figure 4b). This indicates potential sample overloading in the 0.5/2.5 cm breadth spacer. Overloading is caused by excessively high concentrations of sample at the membrane accumulation wall that inhibits full relaxation of the sample components to their equilibrium mean layer thicknesses (l). Indicators of overloading are asymmetric and broad peaks resulting in lower resolution (as observed for the 0.5/2.5 cm breadth spacer). Similar resolution is observed for the 1.0/5.0 cm and 2.0/10 cm breadth spacers, but the 2.0/10 cm spacer has the larger volume and is expected to handle larger sample amounts and was thus chosen for further studies evaluating SP-AF4 channel performance.

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

Sample focusing/relaxation is a unique and important step in AF4. The details of the focusing/relaxation procedure are different depending on the configuration of the AF4 system, but the procedure always involves 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.

Focusing/relaxation is an important step yet the influence of focusing band position of

AF4 separations 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 5a 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 crescent shape for the focusing positions from 2 to 11 cm from the channel inlet (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 ‘upstream’ curvature observed for the 17 cm focusing position. It is also apparent that blue dye is able to move past the focusing inlet and towards the outlet. The ‘downstream’ curvature observed for the 2 to 11 cm bands is due to the triangular end piece utilized at the FFF channel inlet and the subsequent fan of paths traveled by sample as depicted in Figure 5b. The observed curve in the dye front corresponds to 4 cm from the channel inlet. The curvature of the sample band migrating down the channel has been modeled theoretically and observed experimentally in analytical scale channels.[41] However, the curvature of the sample band during the relaxation/focusing step has not been published. The curvature is influenced by the angle of the triangular end piece and wider angles are expected to result in a more curved sample band across the channel breadth.[41] The 2.0/10 cm spacer has the widest inlet angle (118°) of the three spacers examined and would be expected to cause more broadening, but this is not observed in this work. It may be that differences in inlet angles between the spacers (118° , 80° , and 45°) tested are not large enough to cause significantly different band broadening.

Figure 5c demonstrates the effect of focusing position on the separation of PSL standards. As the focusing position is moved further from the channel inlet, the PSL standard shifts towards an earlier t_r . This is expected because the effective channel length (distance from the focusing

position to b_L) is decreased. The void peak intensity also increases while the resolution decreases significantly. The focusing position at 2 cm corresponds to a region within the triangular end piece where the breadth of ~2 cm is similar to that at focusing position 11 cm. However, the effective channel lengths for these two focusing positions are 24.6 cm and 16.6 cm, respectively. The observed difference in resolution suggests that the effective channel length is the main factor influencing the separation. However, differences in the focusing band curvature at different focusing band positions may also influence the separation. Finally, the fractogram obtained for the 17 cm focusing position showed the presence of material eluting before the void peak. This is not surprising based on the observation of the blue dye flowing past the focusing inlet (Figure 5a). The lower resolution and t_r is due to the shorter effective channel length and/or the decrease in channel breadth. Cumulatively, these results suggest that focusing the sample band close to the channel inlet provides the highest resolution. It may also be advantageous to focus closer to b_0 to maximize sample loads in the region near the largest channel breadth.

4.3 Effect of Sample Loading on Resolution

Figure 6a 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 μ g to 100 μ g. In this work, sample loads are increased from 0.4 mg to 20 mg representing an increase of over one order of magnitude. This increased sample mass (20 mg) will result in fractions with concentrations of up to ~1 mg/mL compared to ~0.02 mg/mL for the lower sample load (0.4 mg) depending on the flow rate conditions. The UV absorbance of silica particles is small and even at a relatively high sample loading of 0.4 mg the UV detector response is very low and the resolution can not be accurately determined. A ten times increase in the sample load to 4 mg results in a much higher UV response and the two peaks have a resolution of ~1.3 (Figure 6b). A

further increase to 10 mg sample load yields peaks that are slightly less resolved with only minor overloading effects. For a sample load of 20 mg, the resolution decreases to 0.8. In many cases, this lower resolution is sufficient to produce more narrowly dispersed and relatively pure NP size fractions. The ability to successfully separate several tens of mg of sample in under 30 minutes is a significant advance in throughput and for obtaining sufficient quantities for subsequent NP studies.

4.4 Organic Semi-Preparative Asymmetrical Flow Field-Flow Fractionation

Hybrid nanoparticles (HNPs) are composed of multiple material domains fused together through solid-state interfaces. The combination of distinct inorganic domains can result in particles that exhibit multiple functionalities and/or synergistic properties.[44–50] However, the synthesis of HNPs with narrow size, composition, and morphological distributions is challenging.[6,51] Syntheses of hybrid nanoparticles often require multiple reaction steps where subsequent material domains are added to a pre-synthesized nanoparticle population. Furthermore, multiple nanoparticle morphologies can be obtained during each synthetic step, resulting in even more sample polydispersity. In this work, the size polydispersity of HNPs composed of Pt and Fe_3O_4 domains were analyzed by organic SP-AF4. The particles were synthesized in a two-step reaction sequence, where Pt nanoparticles having diameters of 6-7 nm were first synthesized and then used as seeds in a subsequent reaction to induce the heterogeneous nucleation of Fe_3O_4 nanoparticle domains, to yield Pt- Fe_3O_4 hybrid nanoparticles. This synthesis resulted in a polydisperse population of Pt- Fe_3O_4 hybrid nanoparticle “flowers”, which consist of a central Pt particle interfaced with multiple Fe_3O_4 domains. The wide size and morphological polydispersity of a sample such as this may make it difficult to determine complex structure-function relationships such as enhanced magnetic properties [52] or intra-particle magnetic coupling [53].

Organic SP-AF4 has not been previously demonstrated in published literature. The HNPs used in this work are stable in organic solvents that are incompatible with common aqueous AF4 systems. Figure 7a shows the separation of the Pt-Fe₃O₄ nanoflowers and single domain Fe₃O₄ nanoparticles with each sample injection containing ~0.1 mg of each particle type. At sample loads of 0.02 and 0.1 mg the TEM determined diameters are ~8-15% smaller than the d_h determined by organic SP-AF4 (Table 2). The AF4 determined sizes are larger than the TEM determined sizes, possibly due to the inclusion of the solvent shell in the d_h . Figure 7b shows the fractograms and the root mean square radius (r_{rms}) for the Pt-Fe₃O₄ nanoflowers sample. The increase in r_{rms} as a function of t_r demonstrates the successful size separation and provides the size distribution of the Pt-Fe₃O₄ nanoflowers. Interestingly the r_{rms} distribution has two distinct regions from 7.5 to 20 minutes and from 20 to 35 minutes. The first region has a r_{rms} of ~5 nm to 8 nm while the second region shows an increase in size from ~10 nm to 17 nm. The diameter for the single domain Fe₃O₄ nanoparticles as determined by TEM analysis is 11 nm and for the Pt-Fe₃O₄ nanoflowers is 27 nm, both of which fall inside the 5 nm to 8 nm and 10 to 17 nm r_{rms} size ranges, respectively. The two distinct regions suggest that a population of free Fe₃O₄ particles is present in the Pt-Fe₃O₄ nanoflower sample. The r_{rms} can be related to the geometric radius (r) determined by TEM for spherical particles using the relationship $r_{rms} = 0.77r$ [54] and yields r estimates of ~6 nm to 10 nm and ~13 nm to 22 nm for the two regions. These values are only estimates because the single-domain Fe₃O₄ nanoparticles are near the lower size limit for r_{rms} determination with an uncertainty of ~70% (determined from fluctuations (noise) in the detector signals using Astra 6.1.2 software) and the Pt-Fe₃O₄ nanoflowers are not perfectly spherical.

Table 2 shows the AF4 d_h values (calculated from retention time) for the fractionation of

1 increasing amounts of Pt-Fe₃O₄ nanoflowers. The AF4 d_h values for the Fe₃O₄ and Pt-Fe₃O₄
2 particles at sample loads <~0.1 mg are in agreement with the sizes determined by TEM.
3 However, the shift in AF4 sizes from 30 nm to 18 nm as the sample load increases from ~0.1 mg
4 to ~0.6 mg indicates channel overloading. The HNPs are unable to fully achieve their
5 equilibrium positions and elute earlier than predicted by AF4 theory. HNPs overloading occurs at
6 lower sample amounts than observed for silica nanospheres under aqueous conditions. A w of
7 ~150 μ m was determined for the organic SP-AF4 system compared to a w of ~300 μ m for the
8 aqueous SP-AF4 system. Solvents like THF may cause swelling of the regenerated cellulose
9 membrane resulting in a smaller w and reduced channel volume leading to overloading at lower
10 sample loads of ~0.2 mg. In future studies, membranes with better stability in organic solvents
11 should be used to reduce swelling or larger channel thicknesses can be explored.

13 **Conclusion**

14 The separation and purification of nanomaterials is important for understanding their
15 fundamental properties and large sample quantities are often required for further studies. In this
16 work, SP-AF4 channels are designed to handle milligram sample quantities and their separation
17 performance is investigated. Consideration of channel dimensions suggests that increasing the
18 channel breadth is a viable design route to reduce overloading and improve the separation
19 performance of SP-AF4 channels. Experimental results confirmed that wider breadth channels
20 improved resolution for the analytes and sample loads compared to analytical scale channels.
21 The position of sample focusing band plays an important role in sample retention and resolution.
22 The optimal focusing position is as close to the channel inlet as possible or near the broadest part
23 of the channel (b_0) for large sample loads. 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 may reduce resolution and decrease t_r . However, this level of separation performance may be acceptable for large analyte quantities depending on the desired resolution and if sizes of eluted and collected fractions can be determined via online detection methods (i.e. light scattering). Finally, organic SP-AF4 is demonstrated for the first time to separate HNPs. However, the sample loading amount is lower for the HNPs 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.

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Figure Captions

Figure 1. Geometry of the trapezoidal SP-AF4 channel. Complete home-made SP-AF4 spacer dimensions are given in Table 1.

Figure 2. Plots of the channel velocity ($\langle v \rangle_z$) along L for SP-AF4 channels with a $b_0=10$ cm and different b_L/b_0 ratios). The $\dot{V}_c/\dot{V}_{out}$ is 4.0/2.0.

Figure 3. Fractograms of 46 and 100 nm PSL standards. The $\dot{V}_c/\dot{V}_{out}$ was 1.0/0.5, 2.0/1.0, and 4.0/2.0 for spacers with b_L/b_0 ratios of 0.5 cm/2.5 cm (black line), 1.0 cm/5.0 cm (red line), and 2.0 cm/10.0 cm (blue line), respectively.

Figure 4. Size determination using AF4 retention theory (a) and resolution (b) of 46 and 100 nm PSL standards as a function of channel breadth. Error bars are standard deviations with $n=3$ and are smaller than the symbols if not visibly apparent.

Figure 5. (a) Pictures showing the focusing position of blue dye inside the 2.0 cm/10.0 cm channel. A stainless steel block with circular cut-outs that expose the SP-AF4 channel's Plexiglas top wall allows visual examination of the blue dye band. The numbers over each picture correspond to the distance from the channel inlet. (b) Depiction of the sample band curvature due to the triangular end piece of the SP-AF4 2.0 cm/10.0 cm channel. The solid black lines trace the SP-AF4 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 2.0 cm/10.0 cm channel inlet. The $\dot{V}_c/\dot{V}_{out}$ was 4.0/2.0 for all runs.

Figure 6. Fractograms (a) and resolution (b) of 50 and 100 nm silica NPs with increasing sample amount. The focusing time was 20 minutes, focusing position was at 4 cm, and the $\dot{V}_c/\dot{V}_{out}$ was 4.0/2.0 for all runs. Error bars are standard deviations with $n=3$.

Figure 7. (a) Fractograms of Fe_3O_4 nanoparticles and Pt- Fe_3O_4 nanoflowers. (b) Fractograms of Pt- Fe_3O_4 with the corresponding r_{rms} determined by MALS. The $\dot{V}_c/\dot{V}_{out}$ was 2.0/0.25 and a focusing time was 6 minutes for all runs.

Figure 1

Figure 2

Figure 3

Figure 4a

Figure 4b

Figure 5a

Figure 5b

Figure 5c

Figure 6a

Figure 6b

Figure 7a

Figure 7b

Table 1. Dimensions of the home-made SP-AF4 trapezoidal spacers

b_0 (cm)	b_L (cm)	L (cm)	z_0 (cm)	z_L (cm)	Nominal w (μm)
0.5	2.5	23.6	3.0	1.0	350
1.0	5.0	23.6	3.0	1.0	350
2.5	10.0	23.6	3.0	1.0	350

Table 2. TEM and AF4 determined diameters for the Fe₃O₄ nanoparticles and Pt-Fe₃O₄ nanoflowers

Sample Type	Sample Load (mg)	TEM (nm)	AF4 (nm)
Fe ₃ O ₄	0.10	11	12
Pt-Fe ₃ O ₄	0.02	27	30
Pt-Fe ₃ O ₄	0.10	27	32
Pt-Fe ₃ O ₄	0.20	27	19
Pt-Fe ₃ O ₄	0.60	27	18