

Characterization of protein aggregates and other biological systems using FFF and SPLITT techniques

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THE CHARACTERIZATION OF any sample, especially biological samples, requires a separation step in order to discriminate the various species and contaminants contained within the sample. The most common separation methods are elution based, usually involving chromatographic columns. The benefits of such systems are that the well-characterized fractions are physically separated so that the individual species are quantitated directly and can be collected for further analysis by microscopy or nuclear magnetic resonance (NMR), for example. A more convenient setup may direct the fractions to an on-line detection system, such as an MS, MALS (multiangle light scattering), or viscometric detector. These standalone detectors are currently available with sufficient sophistication so that multidimensional information can be generated from the single analysis. The last advantage of elution-based methods is that automation of the analysis is easy to implement.

Field-flow fractionation (FFF) is an alternate member of the elution-based family of methods,¹ "alternate" meaning that no subordination in performance, utility, or importance is intended. FFF offers all of the advantages mentioned above for elution-based methods. Additionally, FFF serves to complement the current chromatographic methods, providing high-resolution separation of high-MW species. In many situations, FFF can be used where chromatographic methods fail and can also provide superior results for sample types, which are problematic for chromatographic methods. In part, this is due to the open-channel structure used for FFF (which deviates from the packed column structure used in chromatography). While FFF provides an analytical-scale, high-resolution separation in an open-channel format, the open-channel equivalent for larger-scale separation is termed SPLITT for split flow thin cell fractionation.² The advantages of the open channel format for FFF and SPLITT systems are listed in *Table 1*. The FFF system and its application to biological samples is described below, followed by a description of the SPLITT system and its applications.

Materials and methods

FFF channel structure

The typical FFF channel is depicted in *Figure 1*, and an example of a cross-flow FFF (or flow FFF) channel is shown in *Figure 2*. As seen in both of these figures, the cross-sectional shape of the FFF channel is rectangular with a large aspect ratio. This large breadth-to-width ratio ensures a laminar flow profile across the thin dimension of the channel. This well-

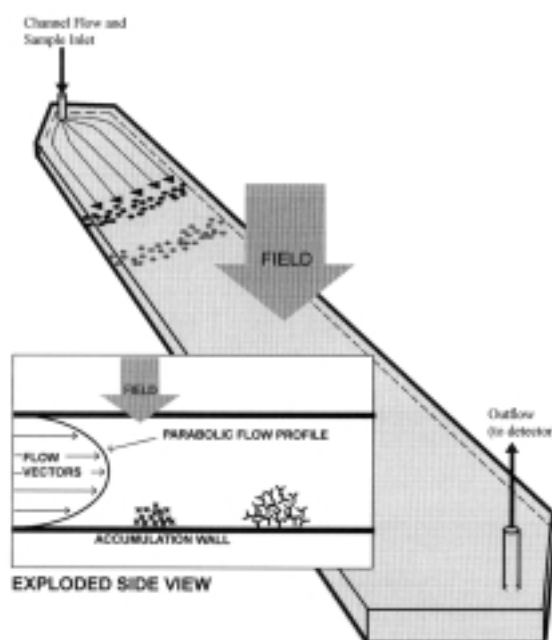


Figure 1 End-on view of the FFF open, rectangular channel. Exploded view depicts the FFF separation mechanism.

controlled flow pattern leads to a well-controlled, reproducible, and reliable separation. Since the FFF channel lacks packing material, other means to drive the separation are necessary. This is provided by an externally applied field. As can be seen by the exploded view, the field is applied perpendicular to the direction of flow in the channel. Different fields may be used so that (as with chromatography) there is a large family of FFF techniques, each member relying on the use of a different field. For biological applications, the most useful fields have been the sedimentation and cross-flow fields. The sedimentation FFF system has been used to separate and characterize the mass of bacteria, viruses, cellular components, and larger species such as blood cells and yeast. The cross-flow field system is described in detail here, since the targeted application is for the separation/characterization of proteins and protein aggregates (see also Refs. 3 and 4 for a more detailed discussion). To implement the cross-flow field used in flow FFF, porous channel walls (typically ceramic frits) are used to form the channel. *Figure 2* is a schematic of the typical channel construction and system setup.

Principles of FFF separation

In the exploded view shown in *Figure 1*, a coarse representation of the FFF is drawn. At this point in the FFF experiment, the field has driven all sample species to the accumulation wall. Sample then back-diffuses toward the center of the channel at a rate related to diffusivity of each sample species. The collection of Xs represents a high MW species with a relatively slower diffusion rate than the Ys. The flow vectors show the laminar flow profile found in rectangular channels, which is key to the FFF process. Since the higher MW species (the Xs) are congregated closer to the accumulation wall where the flow is sluggish, their passage through the channel has been retarded relative to the lower MW species. These highly diffusing species have a dynamic equilibrium-based position that is of a higher elevation in the channel; thus this zone travels at a faster speed through the channel. It should also be noted that all species are retarded relative to nonretained markers. This is another contrast between FFF and gel permeation chromatogra-

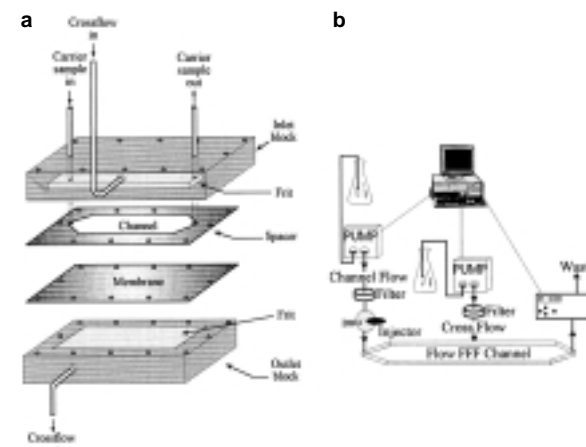


Figure 2 a) Schematic of the flow FFF channel. b) Instrumental setup of the flow FFF system.

phy (GPC): There is no exclusion limit to define the upper limit of MW that can be analyzed. Using the open-channel flow FFF technique, macromolecules and particles can be run in a single analysis due to the relatively unlimited dynamic range of the technique.

Making measurements with FFF

Due to the well-characterized geometry of the FFF channel, in addition to the well-characterized flow properties in the FFF channel, the FFF techniques are measuring systems, not just separation devices. A brief look at FFF theory will illustrate this advantageous aspect.⁵ Since the FFF techniques involve thin, unpacked channels of simple geometry, the flow in these channels is of a laminar, parabolic shape. A simple equation can be written for the linear flow velocity at each position in the channel. Similarly, the field interaction with the sample can be characterized with a simple relationship. This field interaction will determine the average elevation in the channel for each sample species. In the case of flow FFF, the average sample position is determined primarily by the diffusion coefficient of the sample component. A general approximate form of relationship between the retention ratio to these forces can be written:

$$R = 6D/Uw \quad (1)$$

where R is the retention ratio, D is the sample diffusion coefficient, U is the field-induced drift velocity, and w is the channel thickness. For flow FFF, U is equivalent to the linear velocity of the cross-flow stream, and this drift velocity is the same for all sample components regardless of molecular weight. Thus, the retention ratio is then only determined by the diffusion coefficient. Using the Stokes-Einstein equation, the hydrodynamic radius may be calculated directly from the diffusion coefficient. Therefore, from the retention ratio in flow FFF, the hydrodynamic size can be calculated directly and no calibration procedure is necessary. By using the following equation, molecular weight, M , can also be calculated if the constants ϕ and n are available.

$$D = \phi MW^{-n} \quad (2)$$

Lacking these constants, the sample molecular weight can be determined with calibration methods. However, in comparison to GPC, this calibration is needed only to characterize the physical relationship between

Table 1

Advantages of open-channel format used for FFF and SPLITT systems	
Feature	Result
Linear flow velocity of carrier through channel is reduced	Shear rate is reduced; fragile or loosely bound aggregates can be analyzed
No packing material to clog	Samples with particulates as well as macromolecules can be analyzed; no filtration of sample necessary
Fewer material compatibility restrictions	FFF channels have no chromatographic-like packing material; thus a wider range of carrier conditions can be used: pH 3–11; ionic strength; full saline down to deionized water
Surface area contacting sample is reduced	Fewer available sites for adsorption of sample; better recovery and viability of sample

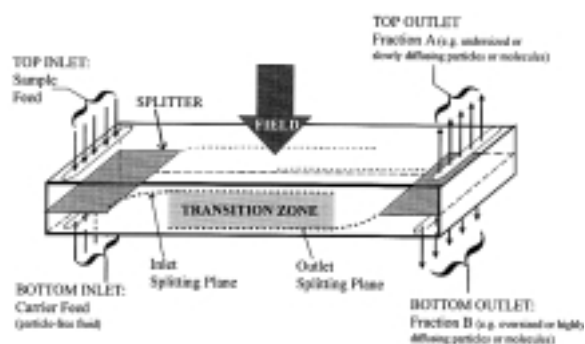


Figure 3 SPLITT channel schematic.

molecular weight and diffusion coefficient. Otherwise, the flow FFF channel requires no calibration as long as channel dimensions are known.

Structure of the SPLITT cell

Figure 3 shows a schematic of a SPLITT cell as well as the principles of its operation. As with the FFF channel, the SPLITT cell is an open structure, rectangular with a wide aspect ratio. Typical dimensions are 4–12 cm in breadth, 20–1000 cm in length, and 200 μm to a few mm in thickness. Often the dimensions are tailored for a specific application, since the throughput and size of the cut-off diameter can be related to channel dimensions.

The most distinguishing feature of the SPLITT cell is the presence of two inlets and two outlets. The function of these ports and the other features marked on Figure 3 is as follows:

- 1) Top inlet: Sample is continuously fed into the SPLITT cell through this port.
- 2) Bottom inlet: Carrier feed is also fed continuously through this port, usually at a higher feed rate than the sample feed. Thus, when the two streams meet, the sample feed stream is compressed into a thin laminae along the top of the channel. The trajectory of the sample is shown by the inlet splitting plane. The ratio of the two inlet flows defines the position of the inlet splitting plane.
- 3) Transition zone: After the sample feed is compressed along the top of the channel, sample species may (or may not) settle through the transition zone. The overall flow rate through the channel may be adjusted so that only certain samples can fully cross the transition zone, for example, samples that are larger than

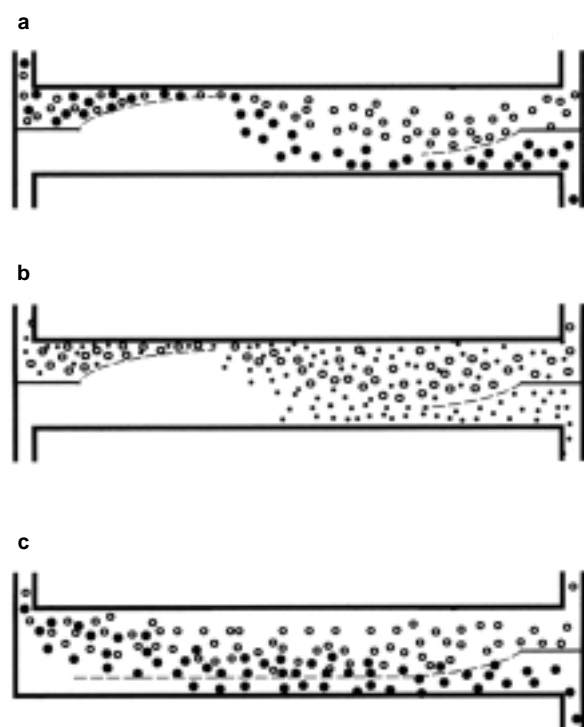


Figure 4 a) Transport mode SPLITT: Heavier particles settle more quickly and exit through the bottom outlet; this is useful for splitting a population into two size-based fractions, or for removal of oversized particles. b) Diffusion mode SPLITT: Small particles or molecules with high diffusivity migrate rapidly versus larger, particulate population; this is useful for obtaining a clean population of molecules. c) Full-feed depletion mode: uses only one inlet, which introduces sample; this is useful for the removal of oversized particles. It also reduces the dilution factor of the final sample fraction.

a certain mass or diameter. Samples that cross the outlet splitting plane will exit from the bottom outlet. Samples with lower migration rates, due to their mass or diffusivity, will remain above the outlet splitting plane and be swept out through the top outlet. By varying the relative flow rates out the top and bottom channel inlets, the position of the outlet splitting plane can be adjusted.

Modes of SPLITT

In addition to varying the type of field applied, the mode of SPLITT can also be varied. The most common is the transport mode shown in Figure 4a. In this case, sample species react to the field by migrating toward the bottom channel wall. The relative rates of migration are critical in determining which species will pass over the outlet splitting plane in order to elute out of the bottom outlet. Simple equations can be written to determine the critical diameter or mass of the particle; this parameter is termed the cut-off diameter. The transport mode is often used to remove oversized particles from a sample. Alternatively, fractions can be collected and reprocessed with a slightly smaller (or larger) cut-off diameter. This exercise generates a narrow size cut of particles.

The migration rate of sample species is controlled by the magnitude and the type of field applied perpendicular to the channel. Most commonly, the Earth's gravitational field has been used for the most simple form of SPLITT. An enhanced gravitational field can be generated by wrapping the SPLITT cell inside a centrifuge basket and spinning the assemblage at hundreds of RPMs. SPLITT systems using electrical and magnetic fields have also been built and demonstrated (see Table 2).

The diffusion mode (Figure 4b) is also interesting for biological applications, especially for drug delivery systems that may contain species differing in size by several orders of magnitude. For example, the amount of drug that has been released or that was not incorporated in a liposome may be determined using this mode of SPLITT fractionation. Table 1 lists the applications of SPLITT references in this work.

Separation classes

The SPLITT techniques excel over other preparative separation techniques when a broad size range is involved. Separation of species differing by 1–16 orders of magnitude is easily accomplished by this family of techniques. Thus, it is useful to categorize the SPLITT separation according to MW ranges. Table 2 also describes this categorization: Class I with a molecular weight range of 10^{12} – 10^{17} might involve supramicron particles such as blood cells, sperm, and yeast. Separation within this class is easily accomplished using the single-gravity SPLITT system. Class II represents sub-micron or 10 million–100 billion MW species. Access-

ing this range requires additional field strength such as that provided with a centrifugal SPLITT system. Class III includes larger proteins and DNA samples. The last class, class IV, designates small proteins, drugs, and contaminants such as surfactants with MWs of less than 1000. By using the diffusion mode or with fields specific to the samples (e.g., electrical SPLITT), species in the class III and class IV range can be fractionated.

Using the various modes of SPLITT, within- and between-class separations can be easily accomplished. Typically, the transport mode is best for a within-class separation and for the larger classes such as I and II, in which diffusion does not compete with the field-induced migration. On the other hand, diffusion can be used to advantage to generate class II or IV samples, which are free of larger particles.

Results and discussion

Resolution of high-MW species

For biological samples such as proteins and protein aggregates, flow FFF is advantageous, especially if higher MWs are involved and/or if a wide size range, such as protein monomers and protein aggregates or particulates, must be analyzed in a single run.

As stated previously, FFF techniques provide higher resolution than chromatographic methods for higher-MW species: For MWs below 50,000 D, GPC performs better. Above 100,000 D, FFF prevails. This claim and the specific limits were theoretically predicted more than 10 years ago. The comparison was reexamined by analyzing a group of protein standards using flow FFF channels and a TSK column set, which was recommended for the MW range of the standards. Figure 5 (top plot GPC; bottom plot flow FFF) show the results of this test. Better resolution was obtained by GPC for proteins smaller than ovalbumin. Specifically, the resolution below the ovalbumin marker is superior to that found with the flow FFF analysis. However, the resolution is superior by flow FFF for proteins larger than this (44K). Moreover, several other species, e.g., bovine serum albumin (BSA) monomer, dimer, and ferritin can fit in between the ovalbumin and the γ -globulin peaks of the flow FFF fractogram. Thus, the effective peak capacity of FFF is greater than GPC, at least for the column set tested.

Analysis of protein and protein aggregates

Using a single channel, both lower- and higher-order aggregates can be analyzed. While in chromatographic methods a change in the column set would be required, with FFF techniques a change in the field parameters (which can be calculated from the simple retention equation) is the only adjustment needed. For flow FFF, changing the field involves changing the flow rate setting of the cross-flow pump. For high-resolution analysis of

Table 2

Applications of SPLITT to biological and nonbiological samples				
Sample	Driving force	Mode	Separation class*	Ref.
Biological applications				
Proteins	Concentration	Diffusion	III/III and III/IV	5–8
	Electrical	Equilibrium	III/III	9
Magnetic particles	Gravitational	Transport	I/I	10
	Magnetic	Transport	I/I	11
Pharmaceutical emulsions	Centrifugal	Transport	II/II	12
Sperm	Gravitational	Transport	I	13
Liposomes and drugs	Gravitational	Diffusion	I/II and II/III	14
Human blood cells, thrombocytes, lymphocytes, and plasma proteins	Centrifugal	Transport	I/III	15
		Diffusion and transport	III/III	16
Proteins and micron-sized particles	Gravitational	Diffusion and transport	I/III	16
Nonbiological applications				
Colloidal particles	Centrifugal	Transport	II/II	17
Starch granules	Gravitational	Transport	I/I	18
Abrasives: silica and diamond particles	Gravitational	Transport	I/I	18, 19
Marine sediments	Gravitational	Transport	I/I	20, 21
Environmental particulates	Gravitational	Full field depletion	I/I and I/II	22
Fuel particulates	Gravitational	Transport	I/I	

*Key to separation classes: I. $M = 10^{12}$ – 10^{17} , 1–50 μm ; II. $M = 10^7$ – 10^{12} , 0.02–1 μm ; III. $M = 10^3$ – 10^7 ; IV. $M = 10$ – 10^3 .

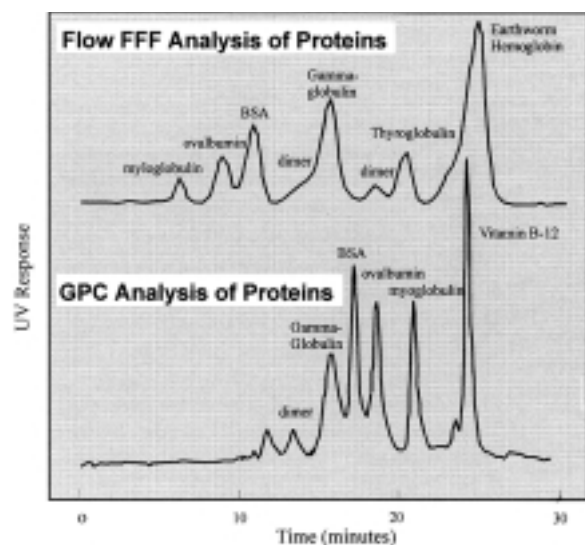


Figure 5 Comparison of flow FFF (top) and GPC (bottom) for analysis of proteins.

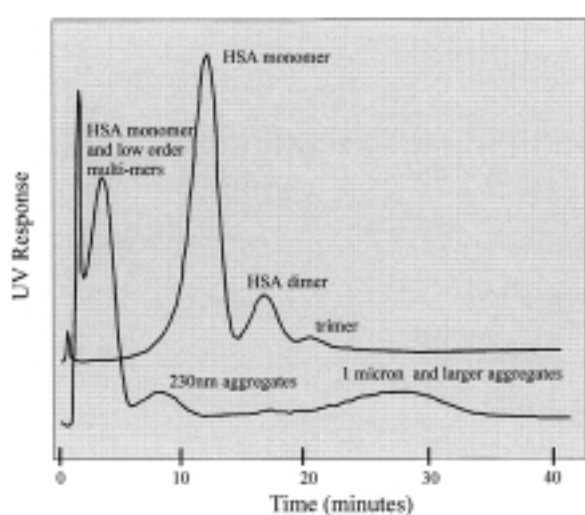


Figure 6 Analysis of human serum albumin aggregates. Analysis of lower-order aggregates (top). Analysis of higher-order aggregates (bottom).

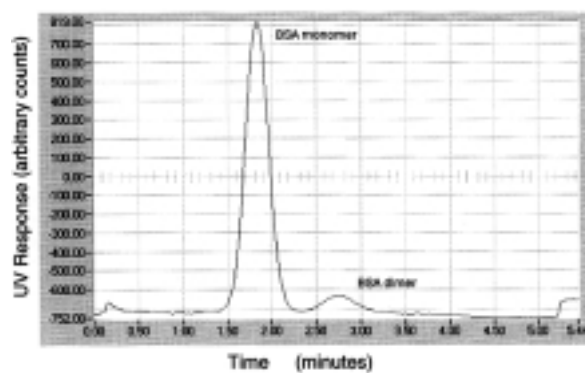


Figure 7 High-speed flow FFF analysis.

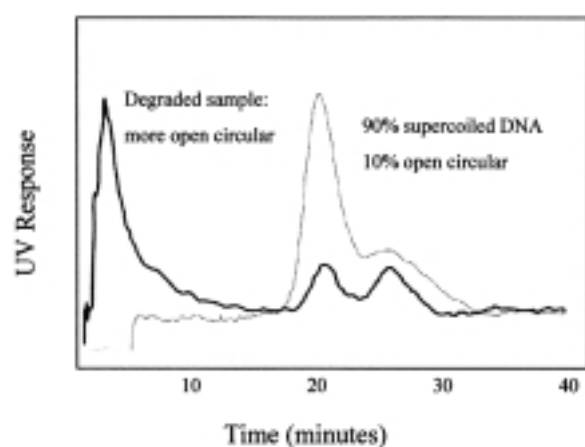


Figure 8 Separation of different forms of DNA by flow FFF.

smaller MW species, a high cross-flow rate is used; for larger MW species, a lower cross flow should be chosen.

Figure 6 shows the analysis of human serum albumin samples, using a high field (top plot) to resolve

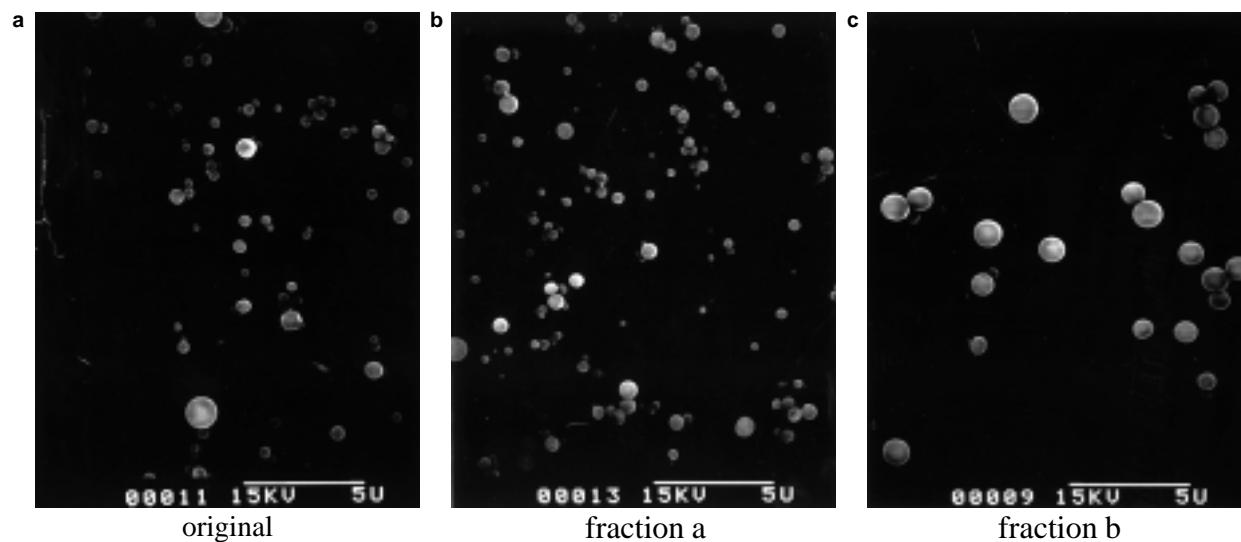


Figure 9 Original and SPLITT fractions of a magnetic particle sample.

the lower-order aggregates. Using a lower field, the larger aggregates could be characterized in a similar analysis time (bottom plot).

Rapid protein analysis

Recent advances in FFF technology have allowed the acceleration of the analysis while maintaining the resolution of the separation. Figure 7 shows recent work using an asymmetric flow FFF channel system. Complete resolution of the monomer and dimer forms of BSA was achieved in less than 4 min.

Separation of DNA conformations

Advances in pharmaceutical science have provided new samples in sizes, shapes, and chemistries that challenge the conventional chromatographic methods. This last example of FFF (Figure 8) shows how the flow FFF technique can be used to separate DNA samples based on the structure of the DNA: The open circular form is less compact than the supercoiled form, and thus separation can be achieved due to differences in diffusivity.

Example application of SPLITT

Recent work done in the authors' laboratory has demonstrated the utility of SPLITT for the separation of magnetic particles. Figure 9a shows the original sample with a reported average size of 0.8 μm . Using FFF techniques, it was found that the range of size extended from 0.1 to 1.3 μm . Using a field strength of 100 gravities, a 2-mL/min sample feed rate, 18-mL/min carrier feed, and 13- and 7-mL/min flow rates for top and bottom outlets, respectively, an approximate cut-off diameter of 0.8 μm was targeted. (Note: The cut-off diameter achieved may vary from the one calculated due to assumptions made about the density of the sample particle.) Figures 9b and c show the clear separation into two size classes. Throughput was on the order of 0.25 g/hr. The goal for this application was to remove oversized particles. However, either fraction could have been reprocessed under conditions for a different cut-off diameter. Then, a narrow-size fraction would have been generated. Typically, the resolution of each cut is +10%. Thus, a fraction that is 0.8–1.0 μm in size can be easily generated.

Conclusion

The last example demonstrates the general SPLITT process. The full power of the technique cannot be illustrated within the limits of this paper. For a better perspective of the utility and range of SPLITT, the reader is directed to the reference list contained in Table 1.

The capabilities of flow FFF are demonstrated by its application to protein aggregate systems and DNA

samples. Although these samples contained species of high MW, even particulate species, the flow FFF technique provided high-resolution separation. In the case of the human serum albumin samples, the wide MW range capabilities were also demonstrated. In a single analysis, monomeric species at a MW of approx. 10^5 D and the particulate aggregates, which were almost 1 μm in diam (which is equivalent to a MW of approx. 10^{11} or a MW range spanning six orders of magnitude), were characterized. Moreover, little sample preparation was required, e.g., no sample filtration was necessary. In comparison to the results of a GPC system, it can be concluded that for biological species of MW greater than 50,000 D, flow FFF provides better resolution. For biological species containing aggregates, the technique is able to characterize both the solubilized species and the particulate aggregates.

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