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FIELD-FLOW FRACTIONATION OF MACROMOLECULES

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SUMMARY

Field-flow fractionation (FFF) is a versatile family of techniques, applicable to macromolecules, colloids, and cell-sized particles. This paper focuses specifically on the applicability of FFF to macromolecules. Following a brief description of the principles of FFF, the characteristics of FFF that bear on its efficacy in separating macromolecules are summarized. The basis of selectivity is established. The general applicability of FFF to macromolecules is then surveyed. For this purpose macromolecular substances are divided into four classes, distinguished by a molecular weight cutoff of 10^6 and by aqueous *versus* organic solubility. The capabilities of different FFF subtechniques in fractionating these classes of macromolecules is then discussed.

INTRODUCTION

The separation of macromolecular materials, both for preparative and analytical purposes, has become one of the most important and demanding activities for separation scientists worldwide. The needs in this area are driven not only by advances in medicine, biology, biotechnology, agriculture, and polymer science but also by increasingly stringent requirements by government regulatory bodies that constantly demand the improved characterization of complex materials of human relevance. Many of these complex materials, particularly in the biosciences, are laden with intricate mixtures of macromolecular components.

In response to the demands for better macromolecular separation and characterization techniques, scientists have responded with a battery of new and vastly improved techniques to separate macromolecules. These techniques include reversed-phase, ion-exchange, affinity, and size-exclusion chromatography; gel, two-dimensional, and capillary zone electrophoresis, along with isoelectric focusing; and the broad field-flow fractionation family of techniques including sedimentation, thermal, and flow field-flow fractionation (FFF). These various approaches are in some respects competitive but in most respects complementary, providing alternate mechanisms for unraveling the formidable complexity of macromolecular materials.

The most recent broad category of macromolecular separation techniques, FFF, is less than a quarter century old[1–3]. Thus, FFF is young relative to chromatography (over 80 years old) and electrophoresis (almost 60 years old). Because FFF is, in

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relative terms, a new methodology not yet in full stride, there is a smaller reservoir of practical experience to draw from than is available for other families of techniques. The level of development and applications work devoted to FFF is only a miniscule fraction of that devoted to the older and better-established groups of techniques. Nonetheless, sufficient work has been done to show that FFF is likely to have an important and substantial niche in the multifaceted repertoire of macromolecular separation tools.

For perspective, we note that FFF has not yet been found particularly effective for low-molecular-weight compounds. The effectiveness of FFF appears to begin at a molecular weight of somewhat less than 1000. From there, the capabilities of FFF, unlike those of most other techniques, improve as molecular mass increases. Thus, FFF is applicable over the entire macromolecular range which, in general terms, can be considered to extend across five or six orders of magnitude in molecular weight. While this mass range will constitute the primary focus of this article, we observe that FFF continues to increase in relative effectiveness beyond the macromolecular limit, proving applicable to colloidal and particulate materials extending over another nine or ten orders of magnitude of mass, extending up to a particle size of *ca.* 100 μm.

The high speed and resolving power of FFF in the separation of the relatively large particulate constituents of colloidal materials and cell-sized (*ca.* 1–50 μm) particles is one of the most important assets of FFF technology as it exists today. By comparison, the development of FFF techniques for the macromolecular range has been somewhat more limited. However, there has been a sufficient accumulation of experience to demonstrate that FFF should have an important role in this area as well.

The nature of FFF has been described on many occasions[2–6]. Briefly, FFF is an elution technique, like chromatography, in which separation occurs within the confines of a narrow tube or channel. There is no packing material and no stationary phase. In the absence of obstructing particles, flow in the tube assumes a uniform laminar profile, usually parabolic in shape. With parabolic flow, the velocity is highest at the channel center and drops to zero upon approaching the channel walls.

Quite obviously, if different species can be somehow placed in different streamlines in the FFF channel, they will be swept along at different velocities and separated. The difficult part in implementing this simple concept is that of finding the means for confining different components to localized regions of the flow cross-section. Such confinement is difficult because entropy-based processes (such as diffusion) are constantly at work distributing entrained components over the entire flow cross-section. Because the channels are thin, any initially confined material tends to spread out rapidly over all streamlines, thus obliterating all velocity differentiation.

In FFF, various driving forces are utilized to force different components to occupy narrow confines of the flow cross-section. These forces must be strong enough to dominate the ubiquitous entropic forces. The driving forces must be oriented perpendicular to flow because their objective is not to drive components along flow lines but rather across flow lines in such a way that they form a unique distribution over the flow cross-section.

For the above purposes many kinds of driving forces have been harnessed, including sedimentation, electrical, thermal (temperature gradient), crossflow, magnetic, and hydrodynamic forces[2–6]. Used singly or in combination, these driving forces are capable of generating different kinds of concentration distributions over the flow

cross-section. The different distributions underlie different operating modes of FFF, including normal FFF, steric FFF, hyperlayer FFF, secondary equilibria FFF, and so on.

The combination of different driving forces and different operating modes leads to a very large family of FFF techniques[7]. Among the prominent subtechniques applicable to macromolecules are those designated as sedimentation/normal FFF, thermal/normal FFF, thermal/hyperlayer FFF, and flow/normal FFF. By this nomenclature the first descriptor (sedimentation, thermal, etc.) refers to the driving force and the second descriptor (normal or hyperlayer) refers to the operating mode[7]. However, as a matter of common practice the word "normal" is usually omitted in describing the normal mode of operation.

The normal operating mode, which has been more widely used than the others, entails the formation of a simple exponential distribution of component molecules or particles against one wall (the accumulation wall) of the FFF channel. Since these exponential distributions are of different thickness for different components, the velocity at which each component is carried along the channel is unique, leading to the desired separation of components.

While the above description of the FFF process and its variations is brief and necessarily incomplete, it provides enough information on the nature of FFF to deduce some of its strengths and weaknesses in dealing with macromolecular materials. By discussing these characteristics, we are better able to deduce the potential role of FFF in macromolecular separations.

CHARACTERISTICS OF FFF

First of all, we recall that FFF, like chromatography, is an elution technique. Techniques based on elution are unusually simple and flexible in sample manipulation, detection, and in general operation. The flexibility of elution is particularly advantageous in the capability it provides for using flow-rate to control the speed of separation over wide limits with no structural changes (*e.g.*, changes in column length) in the separation system. These variations in separation speed generally involve a trade-off with resolution, but the operator has the freedom to choose, with no changes other than in flow-rate, the preferred conditions for separation. We note that some forms of electrophoresis have some quasi-elution characteristics by virtue of electroosmotic flow but these systems have not yet developed the flexibility of true elution systems.

Some differences between FFF and chromatography are centered on the different type of forces used to induce retention. In chromatography, these forces are highly localized at phase boundaries and surfaces. Such forces are highly selective but for macromolecules they tend to be very powerful and are capable of causing irreversible adsorption and structural disruption, including denaturation. The field-based driving forces of FFF and electrophoresis are, by contrast, much more diffuse and locally weak in nature; they rarely reach a level of intensity sufficient to alter molecular conformation.

As molecular size increases, the flow process itself is capable of exerting disruptive shear forces on macromolecules[8]. These forces are particularly harsh in the erratic flow occurring in packed chromatographic beds. By contrast, the shear forces

induced by the uniform laminar flow of FFF channels are relatively gentle.

Operating flexibility is a great asset in macromolecular separations, particularly in exploratory work intended to guage the limits of molecular parameters in a sample. FFF is exceptionally flexible by virtue of the ready variability of the factors underlying the FFF techniques. We have already noted the flow-based flexibility of elution systems, giving immediate access to a range of resolution levels, operating speeds, and sample handling options. Of perhaps even greater importance, retention in FFF is induced by externally controlled forces and gradients that can be altered quickly and precisely to suit experimental needs. Thus, with no change in the FFF channel or equipment, the driving forces can be "tuned" to optimize the separation of components of diverse properties and molecular weights. Not only can these forces be adjusted to maximize the separation of particular species, they can be gradually changed in the course of a run (a technique called field-programmed FFF) to accommodate widely differing sample components[9]. Changes in driving forces also influence (and can be used to control) resolution and speed.

We observe also that most FFF channels have solid, nonpermeable walls compatible with most solvents. Consequently, a given FFF apparatus can usually be adapted to many different solvent (carrier) compositions, thus making it possible to choose a solvent that will maximize the stability and separability of component species. The solvent composition can be rapidly changed for successive runs.

Another important characteristic of FFF is its theoretical tractibility. Because the form (generally parabolic) of the flow profile and the forces exerted on components are controllable and calculable, retention in such systems can be generally related by theory to component properties[2,4]. By linking system behavior and molecular properties, it is possible to control the separation closely. It is also possible to deduce relevant properties of the components from observed retention characteristics.

More difficult to assess is the relative ability of the different families of techniques in resolving macromolecular components. Resolution is related to both selectivity and efficiency[10], the latter reflecting the degree of band broadening in the system. Efficiency in macromolecular separation is generally higher for various electrophoretic techniques than it is for chromatography and FFF. Unfortunately, the high theoretical efficiency calculated for FFF[11] has not yet been realized.

In general, FFF is highly selective. Specifically, it is selective with respect to the particular properties of components that influence the force exerted by the external field. Since different external driving forces can be used, a wide range of selective parameters is available: molecular weight, density, Stokes radius, electrical charge, thermal diffusion coefficient, etc. Separation can be based on any of these parameters by properly choosing the FFF system.

We note that the selective properties of FFF are primarily physical in nature. For FFF, there is little direct selectivity based on chemical properties, which dominate selectivity in most forms of chromatography. (Nonetheless, we note that thermal FFF does show selectivity with respect to the composition of polymeric materials.) In some cases, physical properties (such as electrical charge) are modulated by chemical changes. Electrophoresis, of course, displays a more limited selectivity than FFF based only on electrophoretic mobility; the latter can sometimes be modulated to reflect chemical differences.

Selectivity provides an excellent example of the complementary relationship of different separation techniques. The enormous variation in the basis of selectivity made available by combining all the families of macromolecular separation methods illustrates the magnitude of the arsenal now available to attack macromolecular separation problems and clearly illustrates the complementary role of the different weapons in the arsenal.

FFF APPLICABILITY

Because the FFF family is so broad, it appears that one or more of the FFF subtechniques is potentially applicable to any soluble macromolecule (or suspendable colloid), irrespective of solvent type, presence or absence of electrical charge, random coil or globular conformation, etc. However, in order to examine systematically which of the FFF subtechniques is applicable to any particular macromolecular material, it is necessary to divide the almost infinite variety of macromolecular substances into a few broad categories. It is then possible to specify the FFF subtechniques applicable or partially applicable to each category.

For the above purposes we divide macromolecular components into four classes, as specified below.

(1) Water-soluble macromolecules (WSM) of molecular weight $M < 10^6$.

(2) Water-soluble macromolecules (WSM) with $M > 10^6$.

(3) Organic solvent-soluble macromolecules (OSM) with $M < 10^6$.

(4) Organic solvent-soluble macromolecules (OSM) with $M > 10^6$.

The four categories listed here are not intended to divide all types of macromolecules into rigid compartments. Clearly, many families of macromolecules will span across categories with little regard for either the arbitrary molecular weight cutoff at 10^6 or the solubility criterion that divides one category from another. Nonetheless, most proteins fall cleanly in category 1 while most DNAs fall in category 2. A majority of industrial polymers fall in category 3, and a special group, consisting of ultrahigh-molecular-weight polymers, falls in category 4. Thus, these categories provide a rough grouping of prominent macromolecular materials and make it possible to examine FFF applicability without reference to each of the enormous variety of important macromolecular substances.

Table I provides a summary of the major FFF subtechniques that have been found applicable to each of the four categories of macromolecules. This table is limited to the normal operating mode of FFF. Other operating modes, particularly steric and hyperlayer, are primarily applicable to larger particles, although recent work has shown that a high-speed hyperlayer technique (thermal/hyperlayer FFF) is applicable to category 4 and potentially to category 2 (ref. 12).

Below we describe each of the four subtechniques listed in Table I. We then discuss more specifically the applicability of each subtechnique to the four categories of macromolecules with emphasis on the factors controlling selectivity.

Thermal FFF

The thermal FFF subtechnique is one in which the driving force derives from a strong temperature gradient established between two highly conductive (*e.g.*, copper) bars[4,5]. The temperature drop ΔT between bars, usually 20–80°C, is responsible for

TABLE I

APPLICABILITY OF DIFFERENT FFF SUBTECHNIQUES (IN THE NORMAL OPERATING MODE) TO FOUR CLASSES OF MACROMOLECULAR SUBSTANCES: WATER-SOLUBLE MACROMOLECULES (WSM) AND ORGANIC SOLVENT-SOLUBLE MACROMOLECULES (OSM) OF MOLECULAR WEIGHT, M, EITHER LESS THAN OR GREATER THAN 10^6

× = is or should be fully applicable; + = applicable to some members of class; − = not applicable.

Subtechnique	*1* *WSM* *($M < 10^6$)*	*2* *WSM* *($M > 10^6$)*	*3* *OSM* *($M < 10^6$)*	*4* *OSM* *($M > 10^6$)*
Thermal FFF	+	+	×	×
Sedimentation FFF	−	×	−	+
Flow FFF	×	×	×	×
Electrical FFF	+	+	−	−

extremely high temperature gradients, since it is applied over a very thin gap only *ca.* 100 μm in thickness. Gradients up to *ca.* 10 000°C per centimeter are realized. Changes in the gradient are used to control retention. Commercial instrumentation has recently become available (FFFractionation, Salt Lake City, UT, U.S.A.).

The strong temperature gradients of thermal FFF give rise to a thermal diffusion effect in which components are driven along the temperature gradient, thus sideways across the channel. The driving force of thermal diffusion is particularly strong for nonpolar macromolecules in categories 3 and 4 (ref. 10). However, limited applicability has also been found for the water-soluble species in categories 1 and 2 (ref. 13).

FFF theory has shown that retention in thermal FFF is dependent upon the ratio of D_T/D, where D_T is the thermal diffusion coefficient and D is the ordinary diffusion coefficient[5]. Unfortunately, D_T is one of the most poorly characterized physicochemical properties. However, D_T values can be measured in the course of thermal FFF operation, and a large data base of D_T values has been compiled in our laboratory. As a result, the basis of polymer selectivity in thermal FFF is now fairly well understood[14].

We note that molecular weight is a property of central importance in the characterization of most industrial polymers falling in categories 3 and 4. Thermal FFF provides high selectivity with respect to polymer molecular weight. This selectivity does not originate in D_T, which has been found to be independent of molecular weight, but in D, which varies inversely with molecular weight. We note that size-exclusion chromatography (gel permeation chromatography) also separates macromolecular components on the basis of molecular weight. Somewhat surprisingly, the molecular weight sensitivity of both the FFF and chromatographic techniques originates in the same factor, the hydrodynamic radius or diffusion coefficient[14]. However, the thermal FFF subtechnique is far more selective with respect to molecular weight than is size-exclusion chromatography[10].

The thermal diffusion coefficient D_T, while free of dependence on molecular weight, is sensitive to the chemical composition of both polymer and solvent. Thus, separations can be carried out on the basis of differences in polymer composition. This holds considerable promise for the characterization of copolymers and polymer blends. A dependence of retention on solvent type is also observed, analogous to the

case of liquid chromatography, where the choice of solvent has become useful in enhancing selectivity.

Sedimentation FFF

In sedimentation FFF, the thin separation channel is wrapped around the inside circumference of a centrifuge basket such that the flow axis is everywhere perpendicular to the sedimentation force, as is generally required for FFF operation. The channel assembly can then be spun at different rotation rates in order to control retention in the system[2].

Sedimentation FFF is now the most widely used of all FFF subtechniques. There are presently two commercial instruments available (Du Pont Instruments, Wilmington, DE, U.S.A. and FFFractionation). A vast majority of the applications of sedimentation FFF are in the colloid and particle fields[15].

The application of sedimentation FFF to macromolecules is subject to a basic limitation: the driving force, proportional to molecular mass, is too weak to induce the retention of low-molecular-weight macromolecules. At the highest spin rates available, some retention begins to appear at a molecular weight of about 10^6. Above this transition value, sedimentation FFF becomes a highly selective technique, in theory applicable to most macromolecular materials having large molecular components. However, only a few macromolecular systems in category 2 have been examined. Schallinger *et al.*[16] have separated both DNA species and polyacrylamide by sedimentation FFF. Preliminary work on DNA has also been carried out in our laboratories.

In principle, sedimentation FFF is also applicable to the more massive organic solvent-soluble macromolecules in category 4. However, most sedimentation FFF instrumentation developed to date relies on a rotating seal, subject to damage by organic solvents. Consequently, sedimentation FFF has not yet been applied to this important category of macromolecular materials.

The driving force in sedimentation FFF is, as noted above, directly proportional to molecular mass. Consequently, sedimentation FFF displays a high selectivity with respect to molecular weight. Since the driving force is also dependent upon the difference between the density of the retained component and the carrier liquid (solvent), this technique also displays some density selectivity. This has been demonstrated for colloidal particles and is presumably also valid for macromolecules. The density effect can be modulated by changes in the carrier density.

Flow FFF

The subtechnique of flow FFF is implemented by using a channel having permeable walls. The wall elements are made up of porous or membrane layers. The permeable walls allow fluid to be driven into and across the channel, creating a perpendicular flow of carrier liquid superimposed on the normal axial flow of FFF. The perpendicular flow serves to drive entrained components from one wall of the channel to the other[5].

Flow FFF has the advantage of being the most universal of all FFF subtechniques and perhaps the most universal of all separation techniques. This is because all macromolecules, no matter what category they occupy, are fractionated by virtue of the fact that every imaginable species is displaced by simple flow. The strength

of the driving force is determined by the cross-flow rate, which is controlled, like other FFF driving forces, to adjust retention levels[17]. The fact that flow FFF is shown in Table I to be applicable to all four categories of macromolecules reflects the wide range of applicability of the subtechnique. In our laboratory, we have shown flow FFF to be applicable to protein aggregates, viruses, cells, and a variety of water-soluble polymers. Wahlund and Litzen[18] have recently shown that flow FFF can be applied to the fractionation of DNA and other biological macromolecules.

The principal disadvantage of flow FFF is that thin channels, meeting the rigorous uniformity requirements of FFF systems, are difficult to fabricate with permeable wall materials. Because of this, and also because of some interaction of macromolecules with membranes, the performance of flow FFF systems has not yet reached its theoretical potential. This subtechnique should become increasingly important in the future.

Selectivity in the normal mode of flow FFF is determined by differences in component diffusion coefficients which, in turn, are determined by the Stokes (or hydrodynamic) radius of the macromolecules. Since the Stokes radius is essentially a measurement of molecular size, the flow FFF subtechnique is, above all, size-selective. However, as found in size-exclusion chromatography and thermal FFF, size in a homologous class of macromolecules is merely a reflection of molecular weight; in this sense, flow FFF can be considered to display molecular-weight selectivity. The magnitude of the selectivity is about the same as that of thermal FFF, a value considerably higher than that of the best size-exclusion chromatography column.

Electrical FFF

The electrical field of electrical FFF is applied not only across the FFF channel but also across permeable membrane and porous elements that allow the electrode compartments to be isolated from the FFF channel[5]. Because of wall permeability, the system somewhat resembles that used for flow FFF. However, it has been difficult to achieve effective separations in electrical FFF systems, largely because inadequate attention has been paid to the development of the necessary technology. Earlier work in our laboratories showed that electrical FFF is applicable to proteins; applicability should extend to other charged species as well. The limitation of electrical FFF to charged components is reflected in Table I where electrical FFF is shown as applicable to only some members of the water-soluble macromolecule categories.

Selectivity in electrical FFF is based on differences in the effective electrical charge on a species or, considered in another way, it is dependent upon the ratio of electrophoretic mobility to ordinary diffusion coefficient. Thus, selectivity has some resemblance to that exhibited by electrophoresis, but the two are not identical in nature. Like electrophoresis, selectivity in electrical FFF can be modulated by pH and presumably by chemical factors that influence electrical charge.

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