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Continuous Separation in Split-Flow Thin (SPLITT) Cells: Potential Applications to Biological Materials

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Abstract

We recently described a broad class of techniques in which separation is achieved over a submillimeter path extending along the thin dimension of a special separation cell termed a SPLITT cell. The separation is converted into a continuous process by flow through the cell, and the products are collected with the aid of flow splitters. The separation is rapid and predictable by virtue of the simple geometry of the cell. Separation can be based on differences in sedimentation coefficients, densities, electrophoretic mobilities, isoelectric points, diffusion coefficients, etc., depending on operating details. We describe here the principles of SPLITT cells and summarize our preliminary laboratory findings. We discuss various approaches for utilizing the SPLITT system to separate biological materials with components spanning the mass range from the extremes of biological cells down to that of simple amino acids.

INTRODUCTION

One of the difficulties in developing broadly applicable separation methods for biological materials is the enormous mass range of the distinguishable entities that participate in biological activity. The masses of such species range from approximately 10^2 in molecular weight for small bioactive species up to 10^{17} effective molecular weight and above for many cells. Few methods are applicable, at least without significant difficulties, over more than a small fraction of this substantial range. However, there is a broad class of techniques that we generically label as thin cell (or channel) field-biased techniques that has a proven applica-

bility over most of this size range (*2*). Unfortunately, biological separations have been in the minority of previous applications so that much development work is still required. Nonetheless, the intrinsic strength of the methodology combined with the potential for a very wide variety of biological applications suggests an important role for these techniques in solving difficult separation problems in many areas of the biosciences.

While the thin cell field-biased methods all utilize a thin elongated flow cell across whose thin dimension some field or gradient is applied, this class divides into several very broad families (*2*), two of which we mention here. The more mature and better known of the two is field-flow fractionation (FFF). The FFF methods take advantage of a field-controlled differential migration along the flow axis to carry out small-scale batch separations (*3–5*). The fractionated components, as in chromatography, are eluted one by one from the exit port of the cell or channel. Various properties (e.g., mass, Stokes diameter) of the components can be calcualted from the observed retention times. Resolution and speed are relatively high. Among the biological materials, this method has been applied to whole cells (*6*), subcellular particles (*7*), DNA's (*8*), viruses (*9–11*), proteins (*12–14*), protein aggregates of various sizes (*15*), and cell wall fragments (*16*, *17*). Related materials such as liposomes (*18*, *19*) and albumin spheres (*20*) have also been separated and characterized. The methodology appears capable of a much wider range of applicability with further work.

The second family of thin cell field-biased techniques falls in a category termed split-flow thin (SPLITT) cell methodology. The SPLITT techniques, which as a class are much newer in origin, are designed for continuous separation. In these methods the separation process takes place over the thin dimension of the cell (*1*). Flow splitters are used at both inlet and outlet ends to control the distribution of the incoming feed material and to collect fractionated products. This approach is quite different from FFF separation, which occurs along the principal (flow) axis of the channel. Prototype separations in SPLITT cells have been carried out on cell-sized and larger particles ranging up to 50 μm diameter (*21*). At the other size extreme, mixtures of proteins and low molecular weight contaminants have recently been separated (*22*). The preliminary results of our SPLITT studies are summarized in Table 1; most of the results have not yet been published. Many of the studies have been carried out with standard polystyrene (PS) latex spheres in order to test the operation of the system.

It is clear from Table 1 that various subtechniques of SPLITT methodology are applicable over the entire range between the large particle and small molecule extremes and thus over most of the range

TABLE 1
Summary of SPLITT Studies Successfully Carried Out to Date in Author's Laboratory

Case	Driving force	Separation mode	Materials separated[a]	Separation category	Author's coworkers
1	Gravitation	Transport	PS spheres: 7/10/15 μm	I/I	S. R. Springston and M. N. Myers
2	Gravitation	Transport	Glass microspheres: 1–12 μm	I/I	Y. Gao and M. N. Myers
3	Gravitation	Transport	Fuel particulates: ~1–10 μm	I/I	Y. Gao and B. N. Barman
4	Gravitation	Equilibrium	15 μm PS/5 μm PMMA spheres	I/I	B. N. Barman
5	Centrifugation	Equilibrium	0.82 μm PS/0.59 μm PNMA spheres	II/II	B. N. Barman
6	Centrifugation	Equilibrium	0.37 μm VT-t-BS/0.59 PNMA spheres	II/II	B. N. Barman
7	Concentration gradient	Transport	Proteins/low MW contaminant	III/IV	S. Levin, T. Lenczycki, and P. S. Williams
8	Concentration gradient	Transport	10 μm PS spheres/several proteins	I/III	S. Levin
9	and gravitation	Transport	Whole blood: cells/proteins	I/III	S. Levin
10	Hydrodynamic	Transport	PS spheres: 5/10/20/30/50 μm	I/I	J. Zhang

[a]PS = polystyrene, PMMA = polymethyl methacrylate, VT-t-BS = vinyltoluene-*t*-butylstyrene.

occupied by important biological species. The continuous throughput and low volume (~1 mL) of the separation system makes this approach promising for small-scale bioprocessing applications.

A number of important advantages characterize the thin cell field-biased techniques generally and the SPLITT systems in particular. These advantages include:

(1) The separative mass transport is rapid with a time scale tied to cell thickness, leading to rapid separation and short residence times in the separation cell.
(2) The separation can be fine-tuned by controlling the applied field strength or gradient and the flow rates of the participating substreams.
(3) The simple cell geometry and laminar flow make possible the calculation of separation parameters from first principles.
(4) Different kinds of fields can be applied and different splitting arrangements utilized to widen applicability.
(5) The channel is open and therefore virtually uncloggable by complex particulate materials.
(6) The flow profile along any given line extending across the breadth of the channel is remarkably uniform, leading to high resolution.

The importance of some of these advantages has been stressed in a previous publication (*2*). Other aspects will be discussed below.

SPLITT CELL STRUCTURE AND FUNCTION

While SPLITT cells can assume a number of specific forms, an important case is shown in Fig. 1. The cell shown in this figure utilizes both an inlet and outlet flow splitter. Two flowstreams enter the cell. The upper one, labeled a', carries the feed material. The two substreams merge into a single stream as they move past the edge of the flow splitter. The boundary at which the two substreams make contact is termed the "inlet" splitting plane. No flow currents cross the splitting plane. Therefore, in the absence of mass transport through the fluid, the feed material entering inlet a' will remain in the region above this splitting plane. However, the splitting plane may swerve upward or downward as the flowstream passes beyond the splitter edge, depending upon the relative flow rates in the two incoming streams. Very quickly (within a few cell thicknesses) the splitting plane reaches a steady elevation which is

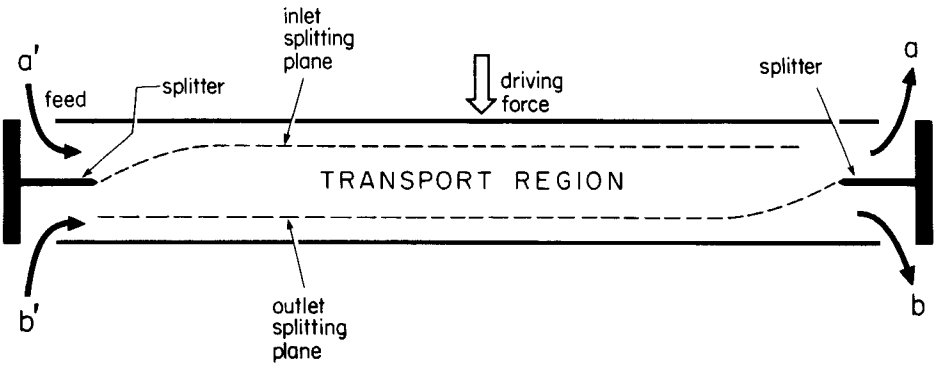

FIG. 1. Edge view of ribbonlike SPLITT cell showing splitters, splitting planes, and transport region within the cell.

controlled by the ratio of incoming streamflows and is independent of the splitter position.

The cell shown in Fig. 1 also has an outlet splitter. Here the single (merged) stream flowing through the body of the cell is divided into two substreams, each with its own exit. The boundary dividing the two outlet streams can be projected back into the channel as a second splitting plane, the "outlet" splitting plane. In the absence of any form of mass transport other than flow, species entrained in the stream above the outlet splitting plane prior to elution will exit from outlet *a* while those flowing beneath the splitting plane will exit outlet *b*. The position of this splitting plane, much like that of the inlet splitting plane, can be adjusted up or down in the channel by varying the ratio of flow rates in the two outlet substreams.

By different adjustments of the four flowstreams (two inlet and two outlet), the inlet splitting plane can be adjusted to a position above, below, or coincident with that of the outlet splitting plane. For the case under consdieration here, this adjustment is crucial. Specifically, the inlet splitting plane must lie above (i.e., on the feed stream side of) the outlet splitting plane. This leaves a thin flowing sheet of liquid between the two splitting planes. This flowing film is termed the transport region (see Fig. 1).

The above relationship between the splitting planes introduces the requirement that any feed stream particle exiting from outlet *b* must first migrate across the transport layer. Consequently, the thin film of liquid constituting the transport region, although lacking distinguishable physical boundaries in its progression through the cell, defines a critical

transport path for separation. Since this path is only a fraction of the thickness of the cell, it will typically represent a distance of only 0.1 mm or so along the cell's transverse coordinate.

The thin transport region resembles in some ways a membrane across which separation is achieved by selective mass transfer. The membrane is continuously generated at one end of the cell and absorbed at the other. Its thickness can be quickly adjusted (by changes in flow rates) and it cannot be fouled.

The crucial mass transfer step, across the transport region in the reference cell described above, can be driven by a variety of forces, including gravitational, centrifugal, and electrical, or by a concentration gradient, in which case it operates as a diffusion (dialysis) cell. Such cells, using gravitational forces, have been shown applicable to particles 7–15 μm in diameter (*21*); the range has been extended down to 1–2 μm in unpublished work (see Table 1, Cases 1–3). In Case 3, glass bead fractions were obtained with coefficients of variation as low as ~10%. Identical cells have been used for the diffusive separation of low molecular weight materials from several proteins (*22*). The theory for these processes has been developed, and the results have been shown to agree very well with those obtained from experimental measurements (*21, 22*).

More generally, SPLITT cell operation can be divided into two fairly broad categories—one, as above, depending for separation upon different transport rates across the transport zone. The other is based upon different transverse equilibrium positions sought by the different particle populations fed into the cell. In the transport mode of operation, different kinds of particles generally have the same final equilibrium distributions (or at least seriously overlapping distributions) across the channel, but advantage is taken of the unequal rates of approaching the equilibrium distributions after the particles are released into the cell from the inlet splitter region.

In the equilibrium mode, advantage is taken of the different equilibrium positions for different species, although the particles can be separated before absolute equilibrium is reached. An example of an equilibrium SPLITT separation is that achieved by a simple sink-float method in which a carrier liquid is chosen intermediate in density between that of two designated particle populations. With such a liquid occupying a cell subject to gravitational or centrifugal forces, the denser particles asssume an equilibrium distribution at or near the lower wall and the less dense particles do likewise at the upper wall. These populations are then physically divided by the outlet splitter and collected from the separate substreams. This technique has been used to separate particles (including those of colloidal size) of different densities

as summarized by Cases 4–6 in Table 1. As in the transport case, the separation based on equilibrium is rapid because of the cell's thinness.

While the equilibrium SPLITT process can be carried out in cells identical to those described above (see Fig. 1), one can also achieve equilibrium separation in SPLITT cells lacking an inlet splitter, although the stream must be split at the outlet for collection purposes. The inlet splitter is not necessary because equilibrium distributions are independent of the initial distribution encountered at the inlet end of the SPLITT cell. However, the achievement of equilibrium can be somewhat hastened by using a split inlet to complement the split outlet.

Not only must inlet splitting be used to introduce a separate feed stream at the beginning of a transport-operated SPLITT cell (in contrast to the lack of such a requirement for an equilibrium-operated cell), but the thinness of the feed stream soon after entry is crucial for resolution. Just beyond (within a few channel thicknesses of) the inlet splitter, at which point the flow laminae and splitting planes have reached a steady flow configuration, the ribbon of fluid constituting the feed stream begins to serve its role as a source region for differential transport. In common with many zonal separation techniques based on differential migration out of a starting zone, resolution is highest when the source zone is thinnest. Therefore, resolution can be enhanced by reducing the flow rate of the feed stream to allow the compression of this stream into a very thin ribbon. However, a thin feed stream is incompatible with high throughput. The tradeoff between resolution and throughput will be addressed in a subsequent paper.

The prototype SPLITT cell illustrated in Fig. 1 is limited to binary stream splitting and therefore to binary sepration because there are only two product streams. This capability can be extended, however, by adding additional stream splitters to further subdivide the outlet flow (*1*). Moving in this direction, transport-based SPLITT systems acquire some resemblance to the Harwell continuous electrophoresis system carried out in an annular gap (*23*). The disadvantages of the multiple splitter approach include the complication of precisely introducing additional splitter elements and the potential loss of reliability in the delicate work of uniformly "peeling" away additional fluid layers. Also, the thickness of the cell invariably increases with the degree of stream subdivision (the gap thickness of the Harwell unit is 5 mm, compared to <0.5 mm for binary SPLITT cells currently in use. Increased thickness has no advantage for throughput but it renders cells more susceptible to flow instabilities and thus to costly remedies as found in the rotational stabilization of the Harwell unit.

In view of the relative simplicity of ribbonlike SPLITT cells with a

single outlet splitter, it is likely that the linkage of such cells will provide greater simplicity and versatility than multiple-outlet cells. Many methods of coupling cells can be imagined and, where necessary, the properties of the carrier liquid can be modified between cells to alter the nature of the separation in subsequent cells. Some cells in a linked network could use gravitational forces while others might be subjected to electrical driving forces. The broad variety of possibilities has been further discussed in the original publication (*1*).

Separation by SPLITT methodology is clearly subject to numerous significant variations. Yet the methodology is defined by just a few basic elements: the thin cell geometry, the flow splitters, and the transverse driving force. The recognition that so many tasks can be done with so few simple elements has surfaced only recently (*1*). However, a number of earlier more specialized systems, developed in isolation from one another, have important areas of similarity to the SPLITT family. Included is the Harwell continuous electrophoresis separator noted above (*23*), based on an original concept by Philpot (*24*). Structurally closer is a thin rectangular cell with binary outlet stream splitting designed for use with thermal diffusion (*25*); unfortunately, thermal diffusion is such a weak driving force that effective separation is difficult to achieve (*26*).

SOME APPROACHES TO BIOLOGICAL SEPARATION

In the Introduction we emphasized the broad size and mass range of biological species requiring fractionation under diverse circumstances. The thin cell field-biased techniques, as noted, are applicable over a major part of this range. Since methods in the SPLITT family have had few applications to biological materials (however, see Cases 8 and 10 in Table 1), we limit ourselves here to the development of some general strategies for separating species within or across different classes of biological materials. These strategies are not exhaustively developed here but rather are intended as examples to illustrate the scope of the technique and to suggest areas of application.

For convenience we divide the material of interest into four arbitrary categories based on molecular weight M:

I. $M = 10^{12}$–10^{17} (~1–50 μm diameter)
II. $M = 10^{7}$–10^{12} (~0.02–1 μm diameter)
III. $M = 10^{3}$–10^{7}
IV. $M = 10$–10^{3}

The mass of a biological species, and thus its rank in these categories, is roughly associated with certain physical parameters capable of influencing SPLITT separation. Thus the sedimentation coefficient decreases from I through IV while the diffusion coefficient increases. Other properties, such as electrophoretic mobility and density, are not significantly correlated with rank.

The approaches listed below are expected to be applicable to biological separation and purification problems categorized according to the ranks of the species that must be resolved. We start with transport-based methods; the application of equilibrium-based approaches will be discussed later.

Most Category I particles are massive enough to sediment rapidly through typical transport zones under the influence of gravity. Thus a simple SPLITT cell like that illustrated in Fig. 1 operating in the gravitational transport mode will fractionate Category I particles (a I/I separation) on the basis of differences in sedimentation coefficients (see Cases 1–3 in Table 1). For example, different populations of biological cells should be separable providing they differ sufficiently (~10–40%) in their sedimentation coefficients. The list of separable bodies should include cell nuclei, platelets, and some cell fragments. This approach would also serve to separate nonbiological contaminants in this mass range from the desired biological entities.

The separation of Class I particles from Class II or Class III species is even simpler. Because of the much higher sedimentation coefficient of particles in Class I, a I/II or a I/III separation can be simply executed by adjusting conditions so that the Class I particles drop through the transport zone and separate rapidly from the Class II and III material. Thus cells or cell-sized particles should be easy to purify with respect to viral contaminants (Class II and III), subcellular particles (generally II), or protein-sized biopolymers (III) (see Case 10). However, a I/IV separation requires caution because the low molecular weight Class IV species diffuse rapidly enough that in some cases they can penetrate through the transport zone by diffusion and contaminate the sedimentation-driven Class I particles. Such contamination can be made negligible by adjustments in flow rates and possibly in cell thickness in accordance with theoretical considerations (*22*).

The separation of species within Class II (a II/II separation) will require that the sedimentation forces be amplified by a centrifuge. While SPLITT cells of the type illustrated in Fig. 1 have not yet been adapted to centrifugal use, a prototype is under construction in our laboratory. In such a device one should not only be able to achieve II/II separation but also II/III separation by selectively sedimenting the Class II materials

through the transport zone. Again, the II/IV separation would require a more careful choice of operating parameters in order to avoid significant diffusive contamination by the Class IV molecules.

The sedimentation coefficient for most Class III components is too small for effective separation in the simple centrifuge units presently envisioned. It is likely, however, that electrical forces can be used for III/III separations. More generally, since electrical forces are so universal, they are promising for separations within each of the four classes outlined above and for any combination of classes. However, one must account for any competing transport processes, particularly transport by diffusion for Class IV species and the gravitational sedimentation of Class I species.

Diffusive separation also gains viability in the lower molecular weight range where sedimentation forces lose effectiveness. With diffusion, Class IV (and some Class III) contaminants can be rapidly stripped away from Class I, II, or III components (Case 8). However, since diffusion is strictly a dissipative process, it is incapable of driving any given component completely across the transport region. While the contaminating residue from any given cell can be depleted in successive cells, this is usually accompanied by dilution of the major components. Nevertheless, the speed of the method should be useful in some applications where species to be separated have large differences in molecular weight and thus in diffusion coefficient.

Other forces and gradients could be used to meet special separation requirements. The possibilities include magnetic forces where a magnetic component requires separation and dielectrical forces where differences in dielectric constant are prominent. These specialized forces may be applicable over no more than a limited range of the four molecular weight categories listed above.

The above transport-based SPLITT separations can generally be carried out in a cell with a single outlet splitter like that illustrated in Fig. 1. This cell permits only binary separation, but higher-order separations can be accomplished in two or more linked cells as noted above. For example, two cell populations could be separated easily from Class II contaminants by gravitational sedimentation in one SPLITT channel after which the two cell types would be swept into another unit and separated from one another by differential sedimentation achieved with careful flow rate adjustments. Alternately, the slower sedimenting cell could be made to remain with the Class II contaminants in the first unit and then be sedimented away from those contaminants in the second unit. The choices are numerous.

The equilibrium-based SPLITT methods are equally versatile. Using sedimentation forces one can, as noted earlier, separate particles of two different densities by using a carrier liquid of an intermediate density. This sink-float technique should be applicable to Class I materials using only gravitational sedimentation (Case 4 in Table 1). Class II materials would generally require a centrifuge. Preliminary results in our laboratory have verified the effectiveness of this approach for nonbiological colloidal particles in the Class II mass range (see Cases 5 and 6). However, such sedimentation schemes cannot in the near future be expected to work effectively in the Class III range; in fact, Class III and IV materials would likely contaminate the otherwise purified products generated from within Classes I and II.

Electrical forces again provide a viable alternative. In this case the separation occurs according to differences in isoelectric point.

As in the transport-based SPLITT systems, other options are available. Particularly intriguing is the case in which crossflow (through semipermeable wall elements) causes a displacement in a direction opposite to that imposed by sedimentation or electrical forces. By playing on the balance between these two opposing driving forces, species could, in theory, be separated on the basis of differences in sedimentation coefficients or electrophoretic mobilities in an equilibrium mode of operation (*27*).

While driving components to two opposing walls is a viable equilibrium approach, useful separations can be expected upon driving all species toward a single wall. The key to this approach is the adjustment of the driving force so that a desired component will be driven strongly against the wall where it will be contained in a thin stratum that can be harvested in a low flow outlet. The contaminating component or components, subject to lesser forces, will be distributed more widely over the cross section of the cell and will emerge primarily from the high flow outlet. While some of the contaminant will remain with the desired component after passage through the cell, a second or a third cell can be linked with the first to achieve higher-order purification. Since the desired component is maintained in a thin lamina by the applied driving force, there will be no dilution of this component for any number of linked cells. (In fact, considerable levels of concentration should be possible.)

While the above approach may be difficult with I/I separation, it should work well in the purification of Class I components from II, III, and IV materials.

CONCLUSIONS

It is clear that a great variety of SPLITT techniques can be applied to the fractionation of biological materials. The advantages and disadvantages of different approaches will need to be sorted out against the challenges of practical problems. While case-specific guidelines will only slowly evolve with experience, some generic advantages and disadvantages of the two main SPLITT categories, based on transport and equilibrium operation, respectively, can be deduced on the basis of general operating principles. However, we note that separation in the two cases generally depends on different parameters (e.g., sedimentation coefficient and density), which suggests that the two approaches may be more complementary (especially when the respective flow cells are linked in series) than competitive.

To start with, it is anticipated that equilibrium-based SPLITT operation will be more robust than transport-based operation. For example, if some obstacle or imperfection in the flow cell causes flow streamlines to deviate locally from their normal trajectories, transport-based separation will be irreparably damaged while equilibrium-based separation will be self-correcting. Likewise, flow perturbations at the edges of the SPLITT cell will interfere with transport-based separation but in most cases will not influence separation in the equilibrium mode. Generally the equilibrium-based approach will be far less susceptible to any depatures from perfect parallel flow in the system.

Because of the sacrifice of throughput necessary to gain resolution in transport-based systems, equilibrium-based SPLITT operation can be considered as generally advantageous as far as throughput is concerned. As discussed above, an inlet splitter is not needed with equilibrium operation; the incoming flow may therefore consist entirely of feed mateiral.

A disadvantage of equilibrium-based separation is that it requires in many cases that some modifier be added to the carrier liquid in order to regulate carrier properties (such as density or pH) in a way that will produce different equilibrium distributions. Transport-based techniques generally do not require such specific carrier properties. The addition of modifiers must be considered with caution for biologial materials because they may interfere with biochemical activity and they may become unwanted contaminants more difficult to remove than those originally targeted.

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