Flow Field–Flow Fractionation: A Versatile New Separation Method

Abstract. *Flow field–flow fractionation is a new separation technique that seems likely to have broad applicability. Its theoretical scope includes any solute for which one can find a solvent and a semipermeable membrane. The principles on which the technique is based are presented. Some experiments with polystyrene beads, viruses, and proteins verify that retention depends solely on diffusion coefficients.*

Using a new type of field-flow fractionation that seems likely to have unusual simplicity and scope for macromolecular separations, we have succeeded in retaining and separating various polystyrene particles, viruses, and proteins. The essential concepts underlying the parent technique may be summarized as follows.

Field-flow fractionation (FFF) is a broad methodology of separation in which solute zones are initially layered at the side of a narrow channel by the application of some external "field" (*1*) (Fig. 1). Layer thicknesses differ for each solute, depending on the interaction between the field and the solute. Solute layers are then displaced by longitudinal flow. Since the flow streamlines are slowest near the wall, solute layers are retarded differentially according to how closely they hug the wall. Fields or gradients so far applied include thermal gradients (thermal FFF) (*2*), centrifugal forces (sedimentation FFF) (*3*), and electrical fields (electrical FFF) (*4*).

The success of each subclass of FFF depends on the strength and specificity of the interaction between the field and the solute. When interaction occurs, solute is transported laterally toward one wall at a velocity U, as shown in Fig. 1. At the steady state, diffusion acts in the opposite direction, along the negative U axis. A steady-state exponential layer is formed whose characteristic thickness ℓ (see Fig. 1) is given by

$$\ell = D/|U| \tag{1}$$

where D is the solute diffusion coefficient or diffusivity. The ratio R of the downstream displacement velocity V of the compressed solute layer to the mean flow velocity $\langle v \rangle$ of solvent has the following theoretical dependence on ℓ and channel thickness w:

$$R = \frac{V}{\langle v \rangle} = 6\left\{\frac{\ell}{w}\left[\coth\left(\frac{w}{2\ell}\right) - \frac{2\ell}{w}\right]\right\} \tag{2}$$

Two basic conditions must clearly be met in order to fractionate a group of soluble compounds:

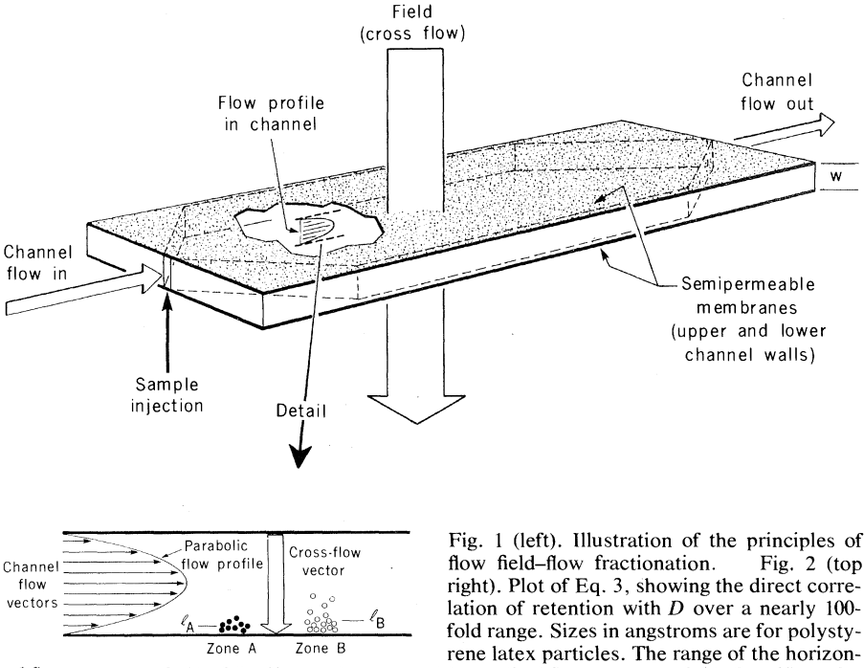
1) The applied field must interact sufficiently with the solutes to form a well-compressed layer and thus retard the solute significantly. Without a reasonable degree of retardation or retention (indicated by small R values), there can be no separation.

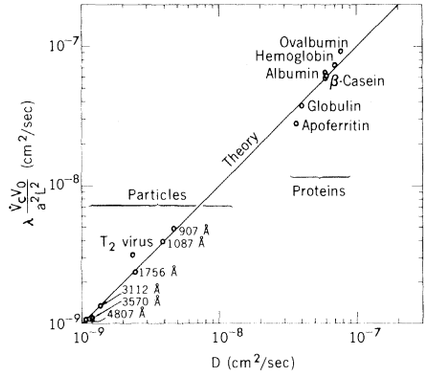
2) Selectivity, or differential displacement, requires unequal values of ℓ/w, or basically, since w is constant, differential values in ℓ and D/U.

In traditional FFF (thermal, sedimentation, or electrical), the first requirement is met only in those selected systems where the field (for example, electrical) is designed to interact with the solutes (charged species). In the system of FFF described here (flow FFF), we substitute a lateral cross flow for the traditional fields and gradients noted above; that is, a bulk movement of solvent from one side of the channel to the other is used to impel solute at velocity U. This lateral motion is superimposed on normal longitudinal stream motion by means of semipermeable channel walls, as illustrated in Fig. 1.

The flow field is unique in that it is absolutely universal: it interacts with all solute species large and small, charged and uncharged. In theory, then, the method is applicable to any solute for which one can find a solvent and a retaining membrane.

Moreover, the flow field affects all solutes with absolute equality, thus failing to impart selectivity like some traditional





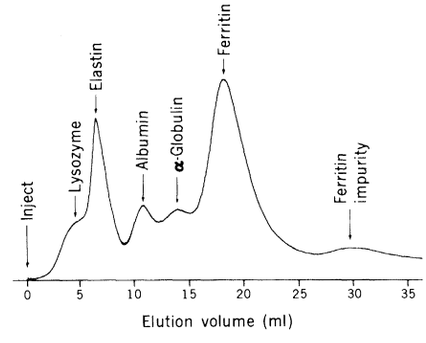


Fig. 1 (left). Illustration of the principles of flow field–flow fractionation. Fig. 2 (top right). Plot of Eq. 3, showing the direct correlation of retention with D over a nearly 100-fold range. Sizes in angstroms are for polystyrene latex particles. The range of the horizontal flow rates was 3.5 to 6.8 ml/hour; the range of the vertical flow rates was 6.4 to 24 ml/hour for the latex beads and virus and 24 to 48 ml/hour for the proteins. Fig. 3 (bottom right). Fractogram of protein components in phosphate buffer, pH 7.13. The horizontal flow rate was 4.65 ml/hour, and the vertical flow rate was 88 ml/hour; the column volume was 1.8 ml.

fields. The second of the above requirements, demanding differential values in ℓ and D/U, must therefore be met entirely by differences in D. Careful considerations show that this requirement is not a disadvantage, and may be useful in correlating retention data with D and in measuring D.

We constructed an apparatus to test this concept. We formed the channel by clamping two membranes together over a stainless steel spacer 0.0254 cm thick from which a flow space 1 by 45 cm had been cut. The upper and lower membranes were cellulose acetate and Amicon UM-20 membrane, respectively. The two flow streams were supplied by metering pumps (Laboratory Data Control model CMP IV). Samples containing about 20 μg of solute were injected at the flow inlet, and the eluted peaks were detected with an ultraviolet detector (Laboratory Data Control). A slightly modified system was used for fractionation.

Positive retention was observed. Furthermore, retention varied from solute to solute, demonstrating a basic fractionating capability of the system.

In order to determine if the retention parameters depend on D in the predicted way, we used the relationships

$$\lambda = \ell/w$$

$$|U| = V_c/aL$$

and

$$V_0 = aLw$$

along with Eq. 1 to derive

$$\frac{\lambda \dot{V}_c V_0}{a^2 L^2} = D \qquad (3)$$

where $\dot{V}_c$ is the volumetric rate of cross flow, V_0 is the channel volume, a is its width, and L is its length. We then plotted the left-hand term of Eq. 3, which depends on observed retention through λ, against independent values of D. For polystyrene beads, we calculated D from the Stokes-Einstein equation; for proteins and viruses we used literature values (*5*). The results are shown in Fig. 2, where the solid line represents the prediction of Eq. 3. The data are thus in basic accord with theory.

The solutes tested here represent an enormous size range, from polystyrene beads with a mean diameter of 4810 Å to β-casein with a molecular weight of 24,100. The mass ratio between these extremes is about 1.5 million. The potential range is even greater as there are no theoretical limits, only practical ones.

An example of fractionation by flow FFF is shown in Fig. 3. We have achieved a resolution or partial resolution of six protein components. Theory suggests that a much better resolution between peaks is possible, but the implementation of this prospect will require additional studies.

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Multiple Sclerosis and High Incidence of a B Lymphocyte Antigen

Abstract. *Multiple sclerosis patients were tested for six new antigens present on human B lymphocytes. The group 4 specificity occurred in 83.9 percent of the 56 patients as compared to 32.5 percent in 72 healthy controls ($P < .003$). The antiserums defining the five other B lymphocyte specificities reacted at a lower frequency to B cells from multiple sclerosis patients, showing that increased reactivity to group 4 antiserums was specific. Linkage of a hypothesized multiple sclerosis susceptibility gene with certain haplotypes of HLA-A3, HLA-B7, HLA-DW2, and the new B group 4 can be inferred.*

We initially suggested the possibility that the HLA region would serve as a genetic marker for susceptibility to multiple sclerosis (MS) by finding that HLA-A3, an A locus antigen, was of higher frequency among MS patients (*1*). This was confirmed by others (*2*), and in addition a B locus antigen, HLA-B7, was shown to be highly associated with MS (*2*). It was next shown that the D locus antigen, DW2 (LD7a), had a higher association with MS (*3*). The inference drawn was that the MS susceptibility gene is probably closer to the D locus than the B or A loci of HLA (*3*). Winchester *et al.* have reported finding a single serum that reacted with the B lymphocytes from 25 MS patients tested (*4*).

B lymphocytes have been shown to have antigens that are distinct from HLA (*5, 6*). The antiserums that react with B lymphocytes but not T lymphocytes have been grouped with respect to their antigenic specificities (*6*). We now describe the typing of B lymphocytes from MS patients for six specificities and the unusually high frequency of group 4 antigen in this disease.

Several centers sent blood from 56 Caucasian MS patients to our laboratory (*7*). Lymphocytes were isolated within 15 hours with the Ficoll-Hypaque density technique and stored in vials in liquid nitrogen. Lymphocytes from 72 healthy control Caucasians were similarly stored in vials in liquid nitrogen. Since HLA frequencies are principally affected by race (and not by age, sex, state of health, or the like), the main effort in matching controls to patients was by restriction to Caucasians.

The B lymphocytes were isolated from blood by removal of T cells by rosetting with neuraminidase-treated sheep red blood cells and centrifugation through Ficoll-Hypaque. These cells were reacted for 30 minutes with a panel of antiserums. After a further 3-hour incubation with rabbit complement, the cells were stained with eosin and fixed with formaldehyde.

The antiserums were selected from more than 400 serums of parous women or patients who had undergone transfusions. The serums were absorbed twice with pooled, packed platelets until all activity against T lymphocytes had been removed. From analysis of the reactions, 33 serums that reacted as six separate specificities were selected (*6*). The HLA-AB locus typing and HLA-DW2 typing were performed by standard methods.

The frequency of reactivity of 33 serums against B lymphocytes of the first 38 MS patients and against controls is given in Fig. 1. The bars represent the difference in frequency of reactivity be-