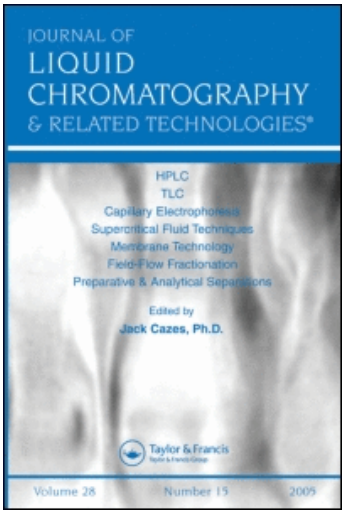

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SEPARATION OF WATER SOLUBLE SYNTHETIC AND BIOLOGICAL MACROMOLECULES BY FLOW FIELD-FLOW FRACTIONATION

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ABSTRACT

Because of the universal applicability of crossflow as a driving force in field-flow fractionation, flow FFF can be utilized for the separation of most classes of macromolecules and particles ranging from less than 1000 molecular weight up to 50 μm particle diameter. This paper focuses on water soluble macromolecules. Among the various types of macromolecular materials recently separated by flow FFF at the Field-Flow Fractionation Research Center and reported here are synthetic water soluble polymers or WSPs (including both anionic and cationic polyelectrolytes) and a variety of biological macromolecules, including low and high molecular weight proteins, protein monomers and dimers, WSP-protein conjugates, lipoproteins, and DNA. These diverse applications can be realized in similar (often identical) channel systems, with only the flowrates needing adjustments to suit the different samples. Since most of these separations take place based on differences in diffusion coefficient or, equivalently, in Stokes diameter, it is a simple matter to correlate experimental retention with the Stokes diameter and measure the Stokes diameter or diameter distribution of the sample. In this paper we will discuss both the specific applications of flow FFF in this area and the general principles and theory that underlie them.

INTRODUCTION

Flow field-flow fractionation (flow FFF) is the most universal of the FFF techniques (1, 2). Because the driving force, acting in a direction perpendicular to the channel axis, is generated by a cross flowstream of carrier liquid, virtually any kind of component, no matter what its size and specific properties, can be driven toward the accumulation wall of the FFF channel, thereby establishing the conditions necessary for successful FFF operation. Both polymers and particles are subject to this mechanism of separation. The lower size limit is currently close to one nanometer. The upper size limit for flow FFF is about 50,000 times larger, 50 μm, a limit that could be further increased if desired.

One potentially important area for the application of flow FFF is the separation and characterization of water soluble macromolecules. This broad category of materials includes many types of synthetic water soluble polymers (WSPs) and an even more diverse cast of biological macromolecules. The object of this chapter is to describe recent results and experiences in applying flow FFF to some of these diverse materials. These results serve to better define the capabilities and future potential of flow FFF in this area.

Our laboratory has been involved in sporadic work on the flow FFF of macromolecules (along with considerably more work on particles) for over 15 years. Among synthetic polymers, we have studied polyacrylic acid and its salts and sulfonated polystyrene (3, 4). Among biological macromolecules, we have focused primarily on proteins and protein aggregates (5, 6). Only recently have other groups started work on flow FFF (7-9). Most notable in the area of water soluble macromolecules is the work of Wahlund and Litzén (8, 9) reporting the separation of proteins, plasmids, and polysaccharides using a form of FFF called asymmetrical flow FFF (10) (see below).

The flow FFF channel is a thin ribbonlike chamber resembling that used in most other FFF techniques. However, the two major walls enclosing the channel are permeable, thus permitting the uniform entry and exit of the cross flowstream. The entry (or depletion) wall is

simply a flat slab of frit. The accumulation wall through which the crossflow exits consists of a membrane layered over another flat frit element. These wall elements are shown in Figure 1. The membrane must be uniform in thickness and have sufficiently small pores that it will retain the components of interest.

Once assembled, the flow FFF system is extremely adaptable. As noted previously, retention is induced by an applied cross flowstream that is capable of displacing virtually all macromolecules and particles over a very broad mass range. The flow rate of this retention inducing stream can be adjusted to suit the mass or size range of the sample components. The mass range that can be effectively resolved in a single run, already quite large, can be expanded further if necessary by using field (crossflow) programming, a technique in which the cross flowrate is varied with time (4). There is no need for carrier (mobile phase) gradients to control component migration as in gradient elution LC. There is no stationary phase and thus little possibility that interaction with a surface will alter or denature macromolecules. There is no packing and thus little tendency to cause the shear degradation of fragile high molecular weight species. The retention mechanism operates within a single phase and is extremely gentle with respect to sensitive macromolecules.

There is always a possibility that some of the sample material will adsorb on the membrane wall. In a similar way, sample may be found to adsorb at the surface of size exclusion chromatography (SEC) supports. However, in SEC the surface area is large, much of it enclosing the molecule-sized pores into which the macromolecules partition. Thus adsorption, if it occurs, can lead to substantial sample loss and, by altering the size and geometry of the pores, it can disturb retention and separation. Adsorption on a flow FFF membrane, by contrast, should create neither of these problems. Because the external surface area of the flat membrane is limited, only a few micrograms of sample will be lost to adsorption even with monolayer coverage (11). If such coverage occurs, it will not measurably influence the retention and separation of samples that are subsequently injected. At worst, there will be a partial blockage of pores leading to an increase in the pressure drop that drives the crossflow through the membrane.

EXPERIMENTAL

Most FFF channel systems, including those of flow FFF, are constructed as a sandwich of multiple layers including wall elements and a thin spacer from which the channel volume is cut and removed. The key layers of flow FFF are illustrated in Figure 1. The frit elements are mounted in clamping blocks. Embedded in the clamping blocks on the back side of each frit (on the side of the frit opposite the FFF channel) are carrier reservoirs to supply and receive the crossflow stream penetrating across the frit slabs. The channel design is very similar to that of the Model F-1000 flow FFF system from FFFractionation, Inc. (Salt Lake City, UT).

The flow FFF devices described above are termed *symmetrical channel systems* because the walls on both sides of the channel are permeable to flow. Such systems differ in construction from asymmetrical systems in which only the accumulation wall is permeable; the depletion wall is normally made of glass or some transparent material allowing the visualization of the channel contents (8-10). Since no cross flow enters through the solid depletion wall, the cross flow exiting the accumulation wall must be provided by the flow stream entering the channel at its normal inlet. Thus the flow at the channel inlet must provide for both the channel flow and the crossflow. The lack of independently controllable crossflow and channel flow streams is the main disadvantage of the asymmetrical variant, a form first developed by Wahlund and Giddings (10). All the work reported here is accomplished using symmetrical channel systems as illustrated in Figure 1.

The experimental work reported below was accomplished using a number of different flow FFF channel systems. The major differences from one system to another are the channel dimensions (specifically the tip to tip channel length L, the breadth b, and the thickness w) and the membrane type used at the accumulation wall. The dimensions of the channels used in this study and the type of associated membranes are tabulated in Table 1. We have also designated in that table that one of the channels is of the frit inlet type, which entails the use of a

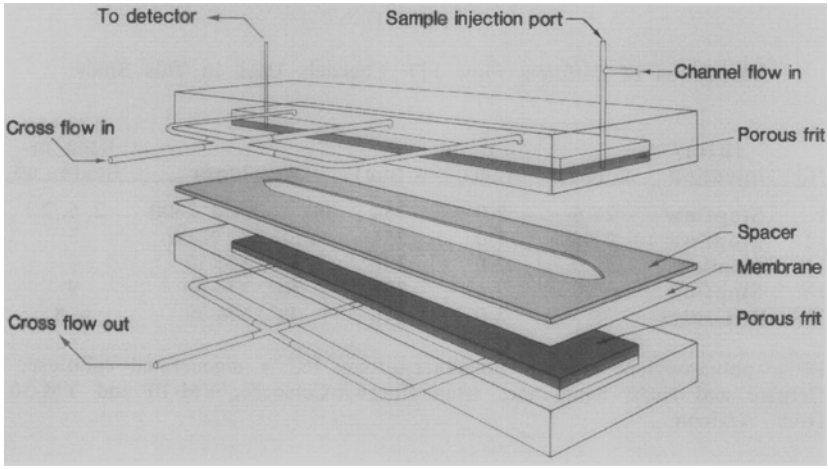

FIGURE 1. Schematic diagram of the structure of a symmetric flow FFF channel.

special channel inlet that allows for sample injection and relaxation without stopflow (12, 13). Without a frit inlet, a stopflow injection procedure must be used. This entails stopping the flow for a brief time t_{sf} following injection to allow sample relaxation.

A large variety of commercial HPLC and peristaltic pumps along with laboratory-built syringe pumps were used in this work. Detection was accomplished using several commercial UV and UV-vis detectors. (The wavelength of detection is indicated with each figure.) Injection was accomplished using an injection valve with a small volume loop or by syringe into a Tee immediately preceding the channel inlet. All injection volumes were 1-10 μL.

THEORY

In the normal mode of flow FFF, applicable to most polymers and colloids, the retention time t_r for well-retained components is approximated by (14)

TABLE I

Description of Different Flow FFF Channels Used in This Study.

No.	Inlet/ injection	L (cm)	b(cm)	w (μm)	Membrane	Used in figure no.
I	Stopflow	27.6	2.0	254	PP, Celgard 2400	2, 5, 7
II	Stopflow	27.6	2.0	254	PES 8K, Nadir	3
III	Stopflow	22.0	2.0	230	RC, YM-10	4
IV	Stopflow	30.0	2.1	227	RC, YM-30	9
V	Frit-inlet	28.5	2.0	170	RC, YM-30	6, 8

PP = polypropylene, PES = polyethersulfone, RC = regenerated cellulose; Celgard and Nadir membranes from Hoechst-Celanese, YM-10 and YM-30 from Amicon.

$$t_r = \frac{w^2}{6D}\frac{\dot{V}_c}{\dot{V}} \tag{1}$$

where w is the channel thickness, D is the component diffusion coefficient, $\dot{V}_c$ is the flowrate of crossflow, and $\dot{V}$ is the flowrate along the axis of the channel. Separation is based on differences in D. However, D can be related to the effective size, specifically, the Stokes (or hydrodynamic) diameter d_s of the component, by the Stokes-Einstein relationship (15)

$$D = \frac{kT}{3\pi\eta d_s} \tag{2}$$

where k is Boltzmann's constant, T is the temperature, and η the viscosity of the carrier liquid. When the D from eq 2 is substituted into eq 1, t_r becomes

$$t_r = \frac{\pi}{2}\frac{\eta w^2}{kT}\frac{\dot{V}_c}{\dot{V}} d_s \tag{3}$$

This equation shows that t_r is approximately proportional to d_s. However, if $\dot{V}_c$ and/or $\dot{V}$ are changed (programmed) during the run, some other (nonlinear) relationship will result (4).

For random coil polymers, d_s is related to molecular weight M by (15)

$$d_s = A'M^b \tag{4}$$

where normally $b \cong 0.6$. (For polyelectrolytes at low ionic strength, charge repulsion will perturb this relationship, effectively creating stiffer polymer chains.) The constant A' depends upon the polymer-solvent system. When these constants are known or can be obtained by calibration, the detector signal versus time curve (the fractogram) can be converted into a molecular weight (or diameter) distribution plot.

We note from eqs 1 and 3 that t_r can be readily controlled by altering the flowrates $\dot{V}_c$ and $\dot{V}$. Thus the system can be readily adjusted to suit the sample, specifically to accommodate the size or molecular weight range of the sample components. In addition, these flowrates can be adjusted to meet analysis speed and resolution goals.

RESULTS AND DISCUSSION

There is little or no difference in basic instrumentation and in the procedure used for synthetic water soluble polymers and for biological macromolecules. Within limits one can employ virtually any kind of solvent or buffer as the carrier liquid so long as a membrane is chosen that is compatible with the solution. For charged flexible chain macromolecules it is best to use moderately high ionic strengths (e.g., 0.1 M) to prevent excessive chain expansion due to charge-charge repulsion within the chain. Expanded chains are more prone to overloading. The flowrates in flow FFF are adjusted to suit the molecular size or weight range without regard for the origin or specific nature of the sample. The primary difference in the treatment of synthetic and biological macromolecules will occur at the sample preparation stage and perhaps in the choice of a wavelength for detection.

Synthetic Water Soluble Polymers

Flow FFF is applicable in principle to any kind of water soluble polymer: anionic, cationic, or neutral. Since cationic polyelectrolytes

are frequently a serious challenge to existing techniques, we illustrate in Figure 1 the flow FFF fractograms of three poly(2-vinylpyridine) or PVP polymer standards. The differential retention of the three polymers and their narrow peaks clearly illustrate the capability of flow FFF to separate these three cationic polymers; the actual separation of a mixture of the three polymers (along with background information on the polymers) was reported in reference 11. An interesting result provided by the individual fractograms of Figure 2 (clearly absent in a fractogram of the polymer mixture) is that the peak for the 240,000 molecular weight PVP standard (and to a lesser extent that for the 110,000 standard) is preceded by a shoulder that suggests the possible existence of a secondary product accompanying the polymer in the main peak. The molecular weight of this shoulder appears to be about half that of the main peak, suggesting (among other possibilities) that the sample may have suffered partial shear degradation. The shoulder is reproducible but was not found in a previous thermal FFF run (11). This observation merits further study.

The runs of Figure 2 were carried out using flow FFF channel I (see Table 1) which employed a polypropylene (Celgard 2400) membrane and a nitric acid carrier solution of pH 1.8. The fractograms bear out the prediction, based on a combination of eqs. (3) and (4), that retention time increases with molecular weight M, an elution sequence opposite to that observed with SEC. The three peaks can be used to generate a calibration plot (11). While the potential resolution of the three PVP polymers demonstrated in Figure 2 is good, much of the observed band broadening is due to the polydispersity of the component samples. Flow FFF can be used to further evaluate this polydispersity (11)

The biggest precaution needed in the flow FFF of water soluble polymers is the avoidance of overloading. The extended chains of these polymers are subject to entanglement and their presence even at low concentrations can modify the local carrier liquid viscosity. These phenomena can alter retention. If the polymers are charged, both their chain expansion and their mutual repulsion can perturb retention. To avoid these effects, one must work at low sample

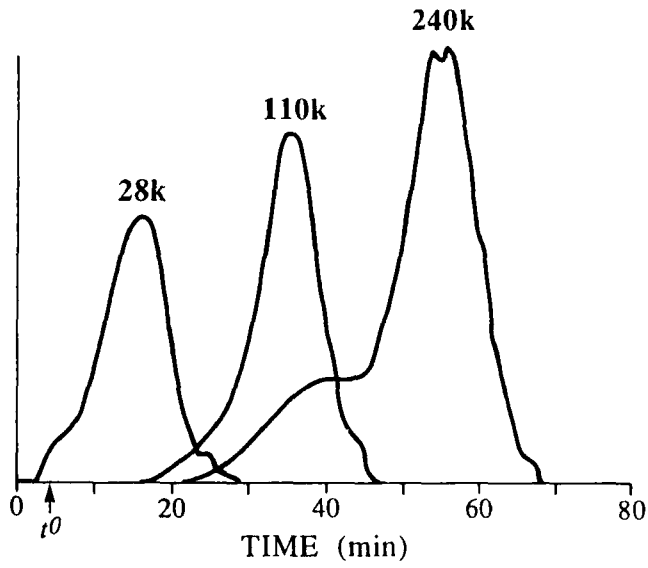

FIGURE 2. Flow FFF fractograms of three PVP polymer standards having the molecular weights shown in the figure. Channel I was used with 0.015 M HNO_3 as carrier. The channel and cross flowrates were $\dot{V}$ = 1.07 and $\dot{V}_c$ = 1.49 mL/min.

concentrations, preferably using submicrogram sample amounts. (The sample loads for the three component peaks of Figure 2 were 0.28, 0.27, and 0.53 µg, respectively.) The requirements will be more demanding as the chain length increases. Consequently, the detector used to monitor the eluent stream must be quite sensitive to properly measure these low concentrations. In the present case, adequate sensitivity was provided by an Applied Biosystems (Ramsey, NJ) Model 757 detector operating at 254 nm wavelength.

As suggested above, flow FFF is an effective technique for many kinds of synthetic water soluble polymers. In a recent reference (11) we have shown that anionic polystyrene sulfonates can be separated at high resolution using the same type of polypropylene membrane as employed for the cationic polymers shown in Figure 2. However, the achievement of separation is *not* linked to the use of *any specific*

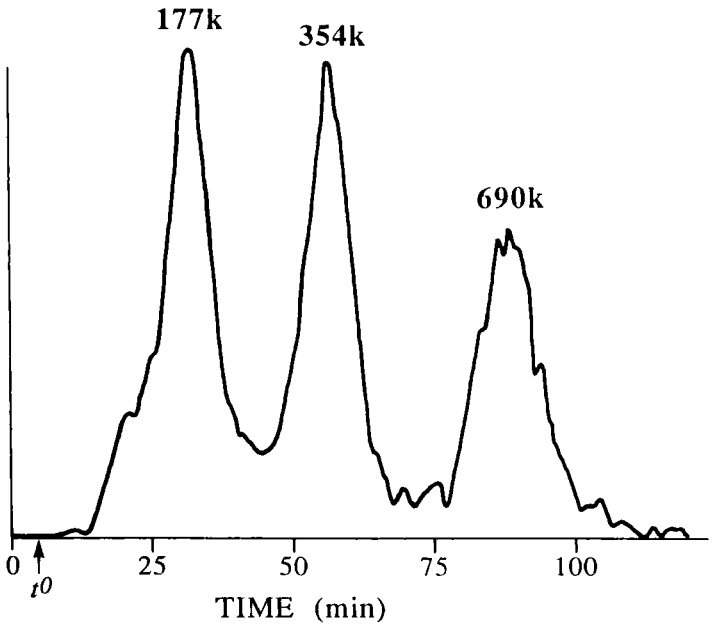

FIGURE 3. Separation of a mixture consisting of nanogram loads (120, 140, 90 ng) of polystyrene sulfonate polymers of the molecular weights indicated in the figure using flow FFF channel II. The flowrates were $\dot{V}$ = 0.30 and $\dot{V}_c$ = 0.71 mL/min. The ionic strength of the carrier, sodium sulfate, was 0.0195 M. Detection at 200 nm.

membrane; membranes of various types should prove suitable providing they are stable in the carrier solution, uniform in permeability and thickness, and that their pores are small enough to retain the polymers. Even if the membrane interacts with the polymer, a monolayer should form after a short conditioning time and from then on, providing the other criteria are met, the system should function properly. While we cannot fully test this hypothesis of interchangeable membranes in this short treatment of synthetic water soluble polymers, we demonstrate in Figure 3 that polystyrene sulfonate polymers can be separated at high resolution in flow FFF using a membrane, polyethersulfone, that differs substantially in virtually all characteristics relative to the polypropylene membranes used previously. The resolution shown in Figure 3 is quite comparable to

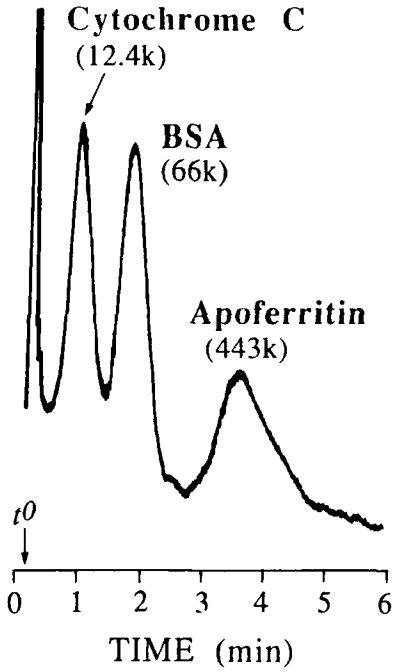

FIGURE 4. High speed separation of proteins by flow FFF channel III. The carrier was a Tris-HNO_3 buffer (I = 0.1 M) at pH = 7.8. The flowrates were $\dot{V}$ = 8.0 mL/min and $\dot{V}_c$ = 6.8 mL/min. Detection at 280 nm.

that reported earlier (11) for the same polystyrene sulfonate standards injected at submicrogram levels into the same carrier solution, 0.0065 M sodium sulfate. (The amounts injected in Figure 3 are 0.12, 0.14, and 0.09 μg, respectively, for the three standards of increasing molecular weight.)

Biological Macromolecules

In the past, our limited applications work on the flow FFF of biopolymers (not including bioparticles such as cells (16)) was focused on the separation of proteins (5, 6). With improvements in instrumentation and techniques, these separations have become faster. Figure 4 shows that the practical time scale for the separation of

proteins by flow FFF has now been reduced to a few minutes. High speed protein separation by asymmetric flow FFF was also reported recently by Litzén and Wahlund (9), confirming that such results are quite generally achievable using various forms of flow FFF.

Perhaps the most important niche for the application of flow FFF to proteins lies in the direction of high molecular weight protein molecules, protein aggregates, various protein complexes and conjugates (e.g., water soluble polymer chains bonded to a protein core), and protein-containing bodies such as lipoproteins. Some of these species have molecular weights extending well into the millions. Our experience in this area suggests that FFF loses none of its effectiveness upon encountering species of such large molecular weight; to the contrary, high mass species are often separated with greater ease than their smaller counterparts.

Some preliminary applications in this high mass area are shown in Figures 5 through 8. Figure 5 shows the separation of the proteins BSA and thyroglobulin, the latter with a molecular weight of 669,000. This separation, completed in under 10 minutes at pH 7.5, utilized the same channel (I) with the same type of membrane (polypropylene) as used for the separation of the synthetic PVP cationic polyelectrolytes at pH = 1.8, shown in Figure 2. This provides further evidence that membranes with suitable mechanical and stability properties can be used for a wide variety of applications.

Since flow FFF is applicable to high molecular weight species, it should be capable of fractionating aggregates of protein molecules, an important topic in the production and use of recombinant proteins. The aggregation process will generate species having a larger Stokes diameter d_s than that of the individual protein molecules, thus leading to an increase in retention time t_r as predicted by eq 3. Thus aggregates of increasing size should be discernible by virtue of their larger observed retention times (6). An illustration of this possibility is shown in Figure 6, which shows the separation of several protein monomers from dimers. In all cases the retention time of the dimer is larger (by 30-40%) than that of the monomer, reflecting the increased size of the dimer species. Similar results have been found by Litzén and Wahlund (9).

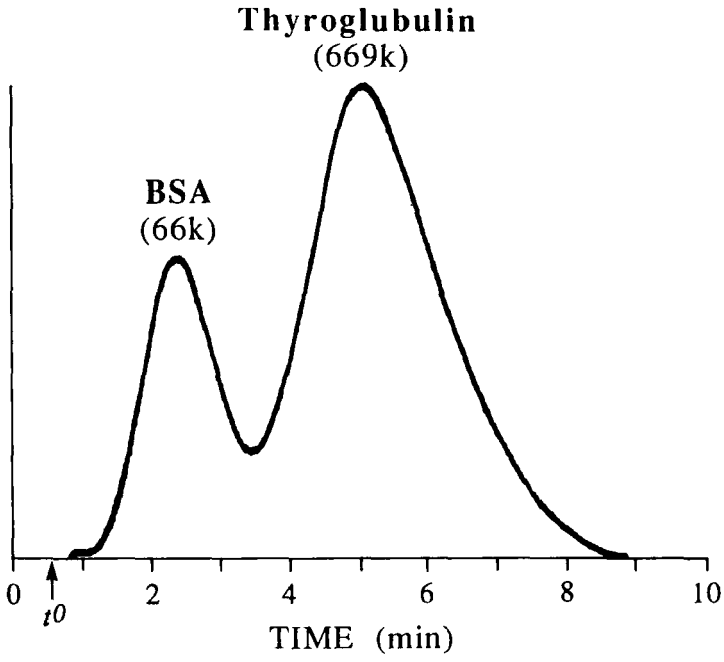

FIGURE 5. Separation of bovine serum albumin and thyroglobulin by flow FFF channel I in a phosphate buffer at pH 7.5. Flow parameters are $\dot{V}$ = 2.0 and $\dot{V}_c$ = 2.03 mL/min. Detection at 280 nm.

Figure 7 illustrates another dimension of high molecular weight protein/biopolymer analysis. In this case we examine the conjugate formed by polyglutamic acid and immunoglobulin IgG. The fractogram on the left for the mixture of unreacted polymer and protein shows two distinct peaks, the first for the polyglutamic acid (~50,000 molecular weight) and the second for the protein. Following conjugation and isolation, the fractogram on the right is observed under identical flow FFF conditions. The latter fractogram is capable of providing a size distribution for the conjugated species. Since eq 3 shows that the retention time is approximately proportional to the Stokes diameter d_s, Figure 7 suggests that the conjugate diameter at the peak maximum is some 2.7 times larger than the Stokes diameter of the immunoglobulin molecule. (A Stokes diameter scale based on eq 3 is provided in Figure 7;

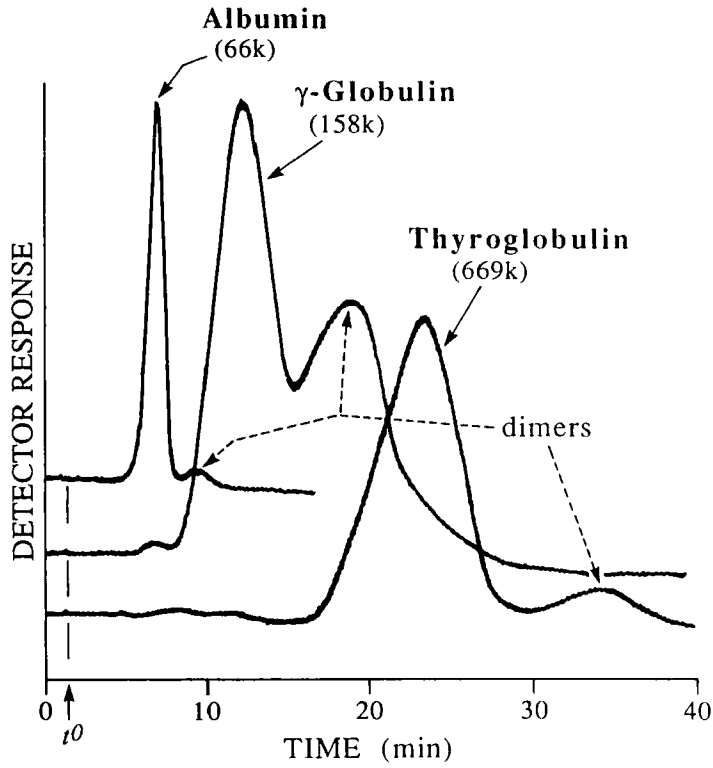

FIGURE 6. Separation of monomers and dimers of common proteins at pH 7.4 using channel V. The flowrates are V = 1.0 and $\dot{V}_c$ = 6.9 mL/min. Detection at 280 nm.

this shows that the respective Stokes diameters of conjugate and protein are 33 and 12 nm.) In view of the growing importance of various conjugates formed between synthetic water soluble polymers and proteins in biomedical applications, the capability of flow FFF to resolve components into different size categories, especially for very large complexes, holds out considerable promise for significant analytical applications.

Proceeding to still more complex protein structures, Figure 8 illustrates the fractionation of lipoproteins from human blood plasma. The fractogram for this material is shown in Figure 8a and the particle

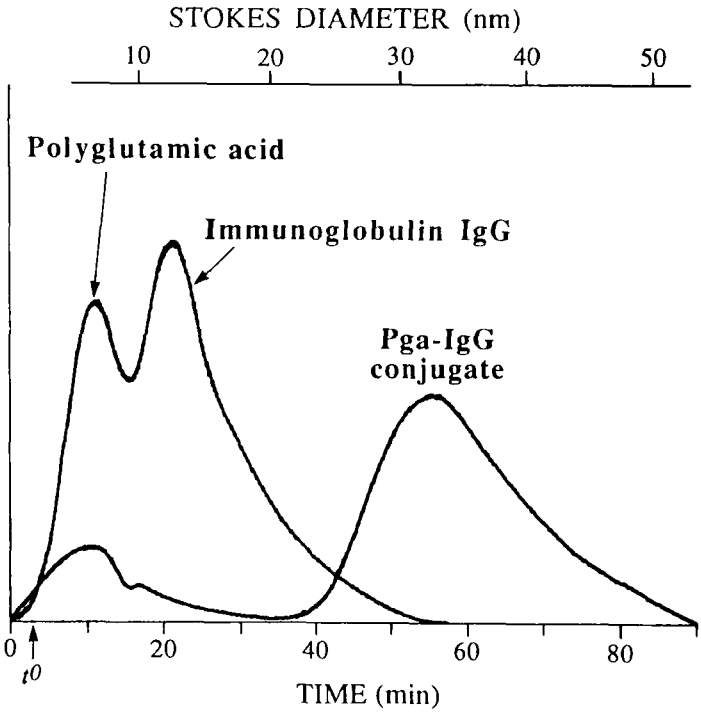

FIGURE 7. Superposition of two fractograms obtained from flow FFF channel I. The two peaks on the left are the components of a mixture of polyglutamic acid (~50,000 molecular weight) and immunoglobulin whereas the fractogram having the large peak to the right was obtained for the covalent conjugate of the previous two components. For both fractograms the carrier was water and the flowrates were $\dot{V}$ = 0.39 and $\dot{V}_c$ = 1.72 mL/min. Detection at 200 nm.

size distribution deduced from this fractogram is shown in 8b. We have shown that distinctly different lipoprotein profiles are obtained for plasma samples from different individuals, providing a characteristic fingerprint for these important constituents and suggesting the possibility for both clinical and research applications. A more detailed study of the application of flow FFF to lipoprotein analysis will be presented in a forthcoming publication.

In addition to proteins and various types of protein complexes, we have recently been successful in fractionating DNA by flow FFF. Figure

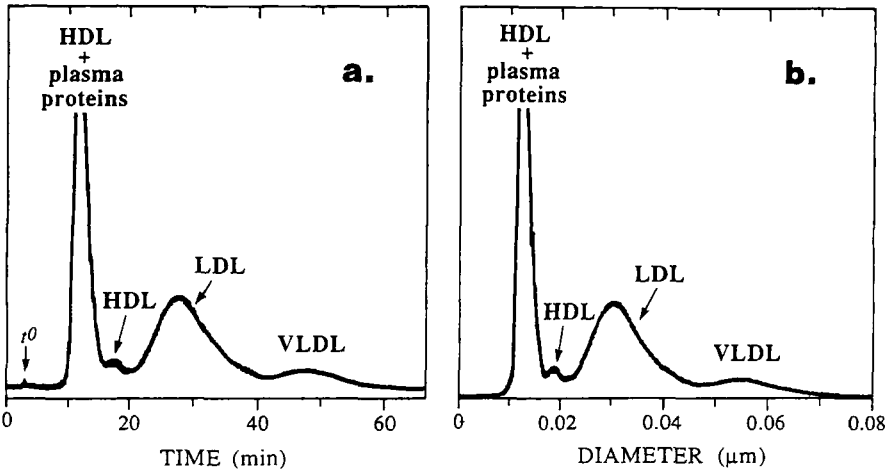

FIGURE 8. Flow FFF of plasma proteins and lipoproteins with conditions adjusted to fractionate the lipoproteins. The run was carried out in a phosphate buffer at pH 7.4 using channel V. The flowrates were $\dot{V} = 1.1$ and $\dot{V}_c = 6.9$ mL/min. The fractogram is shown in (a) and the size distribution obtained from the fractogram is shown in (b). Detection is at 280 nm.

9, for example, shows the separation of the single and double stranded circular DNA components of the ϕX 174 virus. These DNA chains have the same contour length, 5386 bases or base pairs, and thus they have molecular weights differing by a factor of two, 1,777,000 for the single stranded DNA and 3,555,000 for the double stranded DNA. This is the first instance in which the separation of single stranded and double stranded DNA chains has been reported using flow FFF. The separation is almost three times faster and has twice the resolution as reported for these components using sedimentation FFF (17). The observed retention times of these two circular DNA chains are in good agreement with the predictions of eq 1 where D is related to chain length by theory. This suggests that a good correlation can be provided between chain length (for either circular or linear DNA) and measured retention time.

The above examples represent only a small fraction of potential applications of flow FFF to biological materials. Because of the great diversity of biological macromolecules and the challenge they present

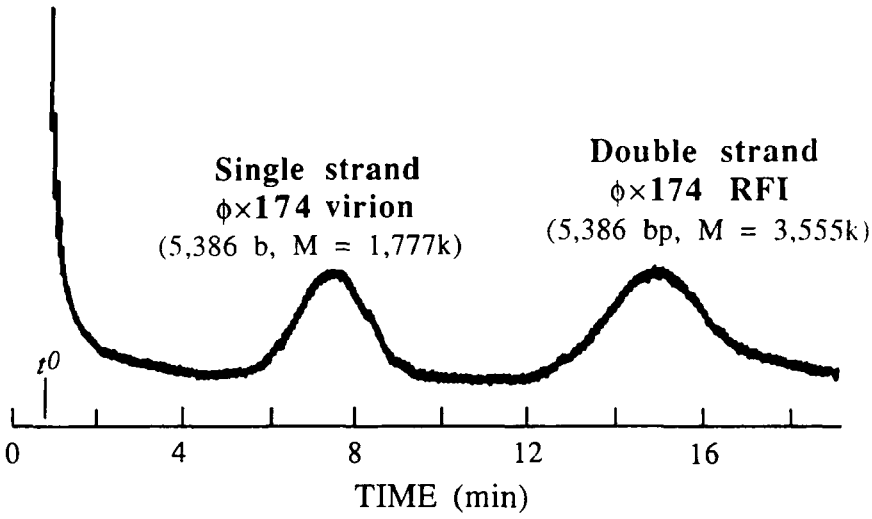

FIGURE 9. Separation of single stranded from double stranded DNA by flow FFF in Tris-HNO_3 buffer (I = 0.1 M) at pH = 7.8. The operating parameters are t_{sf} = 120 s, $\dot{V}$ = 3.15 mL/min, and $\dot{V}_c$ = 1.08 mL/min. Detection at 260 nm.

to analysis, it is anticipated that many more applications of flow FFF will be soon forthcoming in this important area.

CONCLUSIONS

The diverse cast of examples presented above confirms the great breadth of applicability of flow FFF to synthetic water soluble polymers and biological macromolecules and complexes. Most of the results are preliminary in nature; a more complete elaboration in each area will appear in future publications. However a study such as this, focusing on the breadth of applicability of flow FFF, is useful in showing that flow FFF systems of similar or identical structures, operated in some cases with common membranes and subject to similar flowrate adjustments, are applicable to a broad range of water soluble macromolecular materials extending from both cationic and anionic synthetic polymers to proteins and various protein complexes including lipoproteins. A still broader study, which would combine the results of

this report with those of a recent article (18) on the flow FFF of particles, would show that virtually the same flow FFF apparatus is applicable to essentially all macromolecules as well as particles extending up to many micrometers in size. The only significant (but not enormous) variations are those in the two flowrates, which are altered in each case to best satisfy speed and resolution requirements for the individual samples. The common features of this broad base of experimental work provide confirmation that flow FFF is one of the most universal of existing separation techniques.

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