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SEDIMENTATION FIELD FLOW FRACTIONATION AT HIGH FORCE FIELDS

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SUMMARY

New sedimentation field flow fractionation instrumentation with rotor speeds up to 32,000 rpm (about 100,000 *g*) permits separations of materials of somewhat less than 10^6 molecular weight, as well as larger particles. The apparatus has been constructed in an ultracentrifuge with a unique plastic channel rotor assembly. A key feature is that interchangeable plastic sedimentation field flow fractionation channels are mounted within a metal rotor bowl filled with liquid of a specified density. This unique construction allows liquid to surround the plastic rotor assembly, essentially equalizing stresses on the plastic parts at high force fields and permitting low-cost channels to be conveniently constructed. With this design, mechanical stress on the component plastic rotor parts is minimized and specified channel dimensions can be maintained over a wide range of force fields. The new apparatus has been utilized for characterizing a range of materials of biological and industrial interest.

INTRODUCTION

Sedimentation field flow fractionation (SFFF) is a promising method for the high-resolution separation of a wide variety of soluble macromolecules and inorganic and organic colloids and particulates[1–5]. This method can be used to obtain quantitative particle size distributions the 0.005–1-μm range (roughly 10^6–10^{13} molecular weight) where other characterization methods are not satisfactory[6–9]. Most recently, interest in SFFF separations has been in biochemical applications involving low molecular weight materials such as plasmids, viral DNA, and other cell fragments[10].

SFFF separations are carried out in an open channel formed between two closely spaced parallel surfaces. When this channel is rotated in a centrifuge, dissolved or suspended particles that are more dense than the mobile phase migrate toward the outer wall, as illustrated in Fig. 1a. A mobile phase continuously flowing in the channel with a characteristic parabolic velocity profile (Fig. 1b) intercepts these particles near the wall region of slower flow. Smaller particles are, on average, further from the wall than larger particles, as a result of their increased ability to resist the external gravitational force field (Fig. 1b). Therefore, smaller particles are intercepted by faster flow streams and are eluted first (Fig. 1c), followed by particles of increasing mass (Fig. 1d).

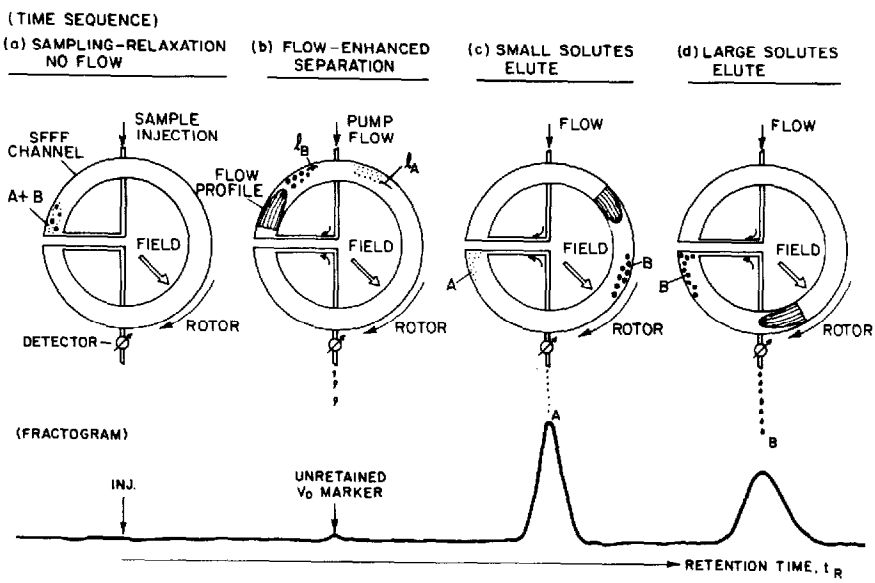

Fig. 1. Development of a SFFF separation.

With constant force-field strength (rotor speed), particle retention in SFFF is related to particle mass or particle diameter according to the relationship:

$$\lambda = \frac{R_0 T}{M\omega^2 r W(\Delta\varrho/\varrho_s)} \tag{1}$$

$$\lambda = \frac{6kT}{\pi d_p^3 \omega^2 r \Delta\varrho} \tag{2}$$

where R_0 = gas constant ($8.31 \cdot 10^7$ g · cm^2/sec^2 · °K · mol); T = absolute temperature (°K); M = molecular weight (g/mole), ω = centrifuge speed (rad/sec), r = radial distance from the centrifuge rotation axis to the SFFF channel (cm), W = channel thickness (cm), $\Delta\varrho$ = density difference between the sample component and the mobile phase (g/cm^3), ϱ_s = density of the sample component (g/cm^3), k = Boltzman constant ($1.38 \cdot 10^{-16}$ g · cm^2/sec^2 · °K), d_p = particle diameter (cm), and λ = a dimensionless retention parameter[1]. For SFFF peaks of practical interest that are well retained and separated from the unretained solvent peak, solute retention increases with decreasing λ value. Eqns. 1 and 2 clearly show that particles are more strongly retained with higher force fields. This means that for components of very small mass or size, very high rotor speeds must be available before sufficient retention can occur for a satisfactory analysis.

Initial SFFF channels were made of metal to withstand the rotor speeds needed for practical SFFF separations. In one approach[1], channels are made of polished stainless-steel bands with metal spacers welded into belt-shaped "sandwiches" suspended in a centrifuge rotor. Fabrication is difficult, and this channel design especially suffers from problems in maintaining constant channel thicknesses (typically

0.025 cm) at high rotor speeds. Welded systems also preclude the ability to expose the channel for cleaning, or to easily change channel configuration.

A second approach initially employed in our laboratories for fabricating SFFF channels[3,6,11] uses a split stainless-steel ring insert fitted into a metal centrifuge bowl with a polished outer wall, to form a SFFF channel that is sealed by "O"-rings to the bowl wall. This split-ring design has the advantage of being able to conform to increases in bowl diameter as rotor speed is increased. With this approach, centrifuge speeds up to 20,000 rpm (about 50,000 *g*) have been used to conduct SFFF separations[4,6]. Unfortunately, split-ring metal channel inserts are difficult to fabricate and expensive. More importantly, leaks across the "O"-ring seals are not uncommon with this design because pressure of the liquid inside the channel can reach 1000–2000 p.s.i. at high force fields.

Another approach for making metal SFFF channels is to use smaller aspect–ratio configurations formed by flattened capillary tubes or by similar methods[12]. Unfortunately, this approach has significant limitations in that moderate force fields can easily distort the channel conformation. Therefore, this method may be limited to relatively low force fields and large particle sizes. In addition, channels with small aspect ratios encourage significant wall effects and secondary flow, which can seriously interfere with the expected retention process.

The present study involves the use of plastic channels in metal centrifuge bowls filled with liquid to minimize density differences between the surrounding medium and the plastic channel. This approach reduces the stress on the plastic and also the pressure difference across the channel seal, and allows centrifuge speeds up to 32,000 r.p.m. with force fields approaching 100,000 *g* without significant channel deformation and seal leakage. In this manner materials of molecular weights of $< 10^6$ can be separated, greatly expanding the applicability of SFFF.

DESIGN CONSIDERATIONS

"Continuous-ring" channel

One of the new approaches for SFFF channels involves a ring with a channel design of a "continuous-ring" type made of an appropriate engineering plastic such as Delrin®* acetal resin or Noryl polyphenylene oxide plastic. As suggested in Fig. 2, this channel is inserted and sealed to the inside polished surface of a metal rotor bowl by lapped bands backed-up with an "O"-ring seal. The outside of the plastic ring and the inside of the metal bowl are made with a diametrical interference fit so that the ring is in compressive contact with the bowl under static conditions. Because of inherent elasticity, this plastic ring can "enlarge" with a titanium centrifuge bowl as the force field is increased.

An important aspect of this design is that the rotor assembly is filled with liquid to minimize density differences between this surrounding liquid and the plastic ring, and between this liquid and the mobile phase liquid inside the channel. In this manner pressure stresses on the plastic channel and on the "O"-ring seal are greatly reduced. The density-compensating liquid permits the use of modest-strength, low-cost plastics for the rotor.

* DuPont registered trademark for acetal resin.

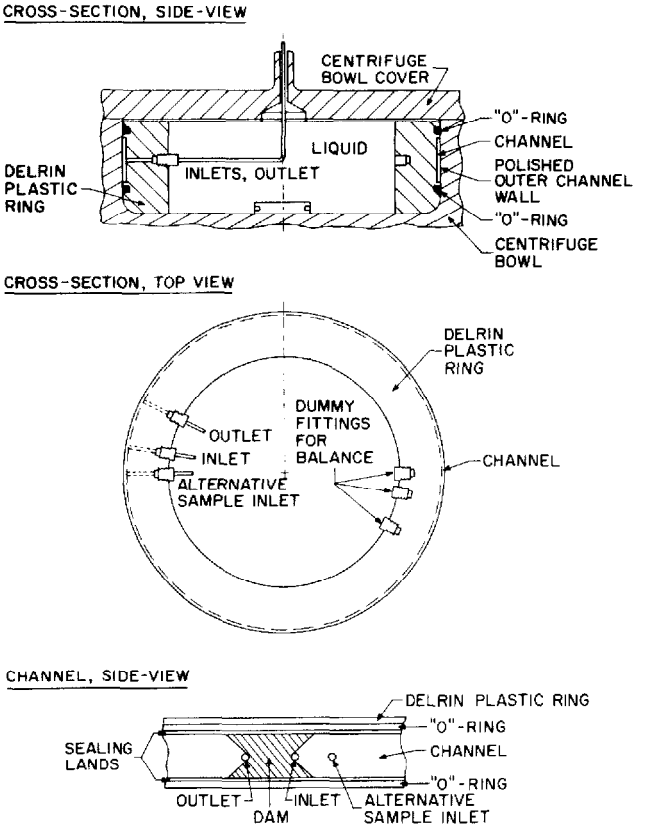

Fig. 2. "Continuous" plastic ring channel rotor for SFFF.

Liquid for filling the centrifuge bowl volume outside of the plastic ring is selected to have a density that is about equal to that used in the channel for the separation. Fluids with densities in the 0.6–1.2 g/cm^3 range may be utilized, depending on the particular application and the requirement that channel dimensions remain constant while the channel is subjected to the force field.

Although in our study the "continuous" ring was made of Delrin, it may be formed of any suitable engineering plastic that is chemically inert, strong, and appropriately resilient. One of the main criteria used for selecting a plastic for this application at high force fields is that its effective density, φ, to tensile modulus, E, ratio generally should exceed that of the material forming the centrifuge rotor bowl. The effective density, φ, is the density of the plastic minus the density of the bowl-filling liquid. With this approach, as the bowl rotor expands under the influence of the centrifugal force, the plastic ring containing the channel expands outwardly in a like or slightly greater amount to maintain contact between the sealing lands and the polished inner surface of the centrifuge bowl wall. In this manner the channel will maintain a leak-free condition throughout the force-field range utilized. For this application a titanium centrifuge bowl is preferred for high rotor speeds. Both the centrifuge rotor bowl and the plastic inner ring materials are selected so that the

dimensions of the channel can be closely maintained throughout the force-field range to be utilized, to minimize uncertainties in the SFFF separation process.

As indicated in Fig. 2, the channel is formed by forming a groove on the outside of the plastic ring that fits inside the centrifuge bowl. The ends of the channel are defined by a dam or barrier (bottom of Fig. 2) formed of material such as Delrin or Mylar® polyester film glued in position to form the ends of the channel. The dam is cut V-shaped at each end so that dead spaces in the flow path of the liquid medium are reduced. An alternative sample inlet port is supplied for special studies.

The continuous-ring plastic channel depicted in Fig. 2 was constructed of Delrin-150 plastic with an inner diameter of 13.005 cm, an outer diameter of 17.882 cm, and an axial width of 5.080 cm, with a lower corner rounded to a 0.9525 cm radius to fit in the centrifuge bowl. This channel had a radial depth of 0.0254 cm and a span of 2.54 cm. It was fitted into a Beckman Model CF-32Ti zonal centrifuge rotor bowl whose inner wall had been carefully machined to a flat finish and highly polished surface. Speeds up to 32,000 r.p.m. (about 100,000 *g*) were obtained without leakage of fluid from the channel.

"*Floating*" *channel*

A more flexible approach is a "floating" plastic channel design in which the channel assembly is totally immersed in liquid contained in a centrifuge bowl. As illustrated by the schematic in Fig. 3, the SFFF channel is formed by an inner hub of Delrin and a mating outer channel ring of Noryl positioned inside a centrifuge bowl. The hub and the outer channel ring are formed to have a diametrical interference fit

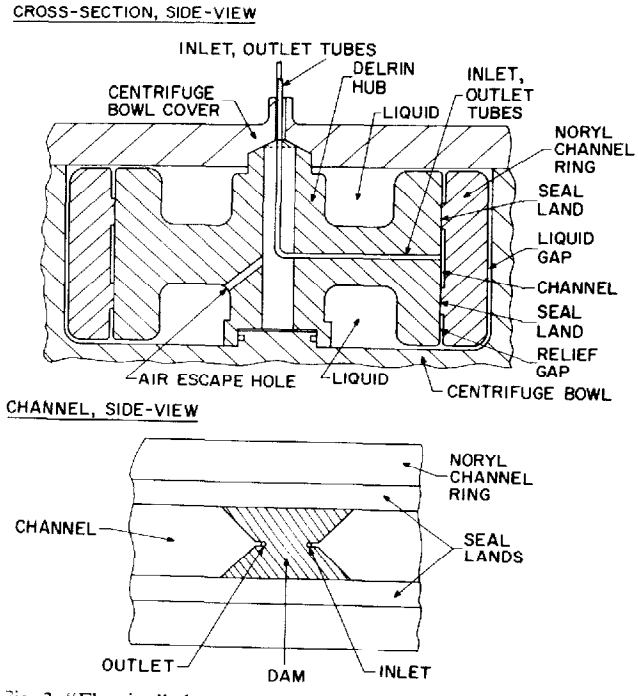

Fig. 3. "Floating" channel rotor for Beckman CF-32Ti bowl.

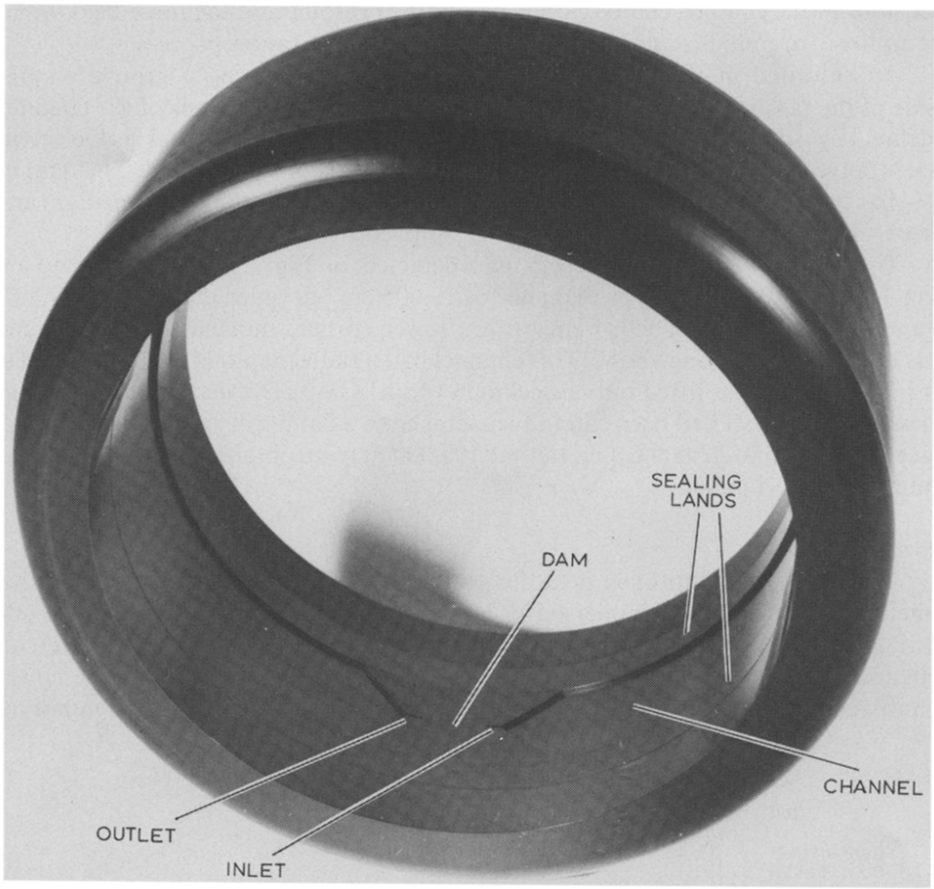

Fig. 4. "Floating" outer ring with SFFF channel.

so that the outer channel ring is in a compressive contact with the hub under static conditions. The separating channel is formed on the inner or mating surface of the outer Noryl channel ring by means of bands or lands on either side of the channel groove which form a seal. The beginning and end of the channels are constructed with a dam by the same approach utilized for the "continuous-ring" channel design (Fig. 2). This dam is very slightly thicker than the depth of the channel groove so that when it is compressed by the smooth outer peripheral surface of the hub, it seals and defines the beginning and end of the channel. Fig. 4 is a photograph of the outer (Noryl) ring containing the channel, in this case, a relatively thick channel of *ca.* 1.0 mm. The inlet and outlet passages as well as the dam and sealing lands forming the channel are clearly defined.

As with the "continuous-ring" design, the interior of the "floating"-type rotor also is filled with a liquid of approximately the same density as the fluid medium that is forced through the channel for separation. The outer Noryl channel ring is fabricated to have a diameter slightly less than the internal diameter of the centrifuge bowl so that the ring does *not* contact the bowl during centrifugation and liquid remains

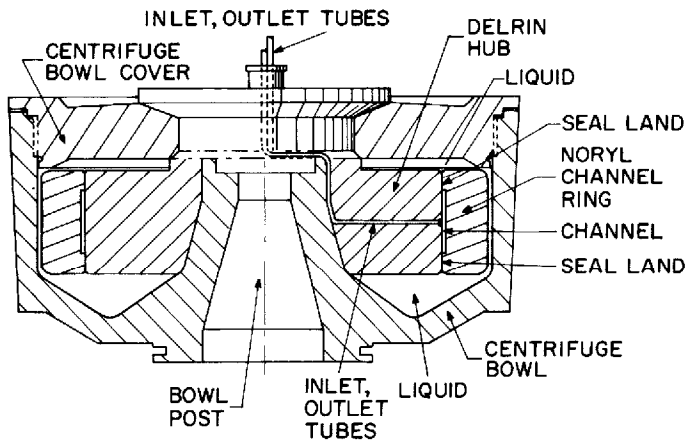

Fig. 5. "Floating" channel rotor for Sorvall TZ-28 bowl.

totally around the assembly —thus, a "floating" channel. The Delrin hub is configured so that it is securely mounted within the bowl. The mid-portion of the hub for a Beckman bowl (Fig. 3) is in the form of an annulus having a reduced thickness to facilitate the outward radial expansion of the hub during centrifugation. This is to insure the required expansion of the hub to maintain channel seal land contact during expansion of the outer Noryl channel ring under increasing force fields.

With this "floating" design, liquid, typically water or a water-based system, surrounds all of the channel assembly including hub and outer ring. Under these conditions when the assembly is rotated, centrifugal force causes the pressure exerted by the liquid in the rotor bowl external to the channel to be essentially equal to that exerted internally by the fluid within the separating channel. For this reason, pressure drops across the channel seal are very low and leakage is essentially eliminated at the interface between the hub and the outer ring. In addition, since mechanical stress on the entire channel assembly is greatly reduced, use of low-cost plastics is permitted.

In this design the channel assembly parts are constructed of plastics selected and designed so that the plastic hub can expand outwardly to a greater extent than the plastic outer channel ring, to insure good contact during centrifugation and thereby maintain the integrity of channel dimensions. As with the "continuous-ring" design, when the ratio of effective density, φ, to tensile modulus, E, of the outer ring is less than that of the hub, the hub expands under centrifugal force at a faster rate than the outer ring, maintaining good contact during centrifugation.

In this design the density of the outer ring is selected to be less than that of the density of the hub. For this reason the density of the surrounding liquid can be different from the density of the fluid within the channel, as long as it is in a range between the densities of the hub and the outer ring. The density of the surrounding liquid within the rotor bowl is selected to be approximately equal to the density of the outer ring. With the design used in the channels schematically shown in Figs. 3 and 5, it is feasible to change the density of the separating mobile phase by about ± 0.2

g/cm^3 and still maintain a good seal between the outer ring and the hub without significant distortion of channel dimensions at high force fields.

Another criterion for selecting the plastics for both the "continuous-ring" and "floating" design is that the surface of the plastic itself must be capable of being polished to a mirror finish. In addition it is also desirable that the plastics used in the channel assembly should be chemically inert, have a relatively high yield strength, and be biologically non-toxic. These requirements were fulfilled by Delrin and Noryl, although other plastics might also be used. It was also required that mechanical techniques be developed so that these plastics could be machined to tolerances of $\pm 2.5 \cdot 10^{-4}$ cm. (1–2 ten-thousandths of an inch) so that the required precision of channel dimension could be obtained.

With the design shown in Fig. 3, the outer Noryl channel ring had an outside diameter of 17.475 cm to clear the inside bowl diameter of a Beckman Model CF-32Ti rotor (inside diameter of 17.792 cm), an axial width of 7.478 cm with a rectangular groove of 2.54 cm in axial span, and a 0.025 cm radial depth to form the SFFF channel with lands 0.953 cm in axial width. In this case the hub was 15.818 cm in diameter and the overall axial height was 8.611 cm. This rotor has been successfully operated to about 85,000 *g* for long periods.

The design illustrated in Fig. 5 was incorporated in a Sorvall TZ-28 zonal rotor for the DuPont RC-5 Superspeed centrifuge. While this channel design has somewhat different dimensions than that for the Beckman bowl, the basic construction of the assembly was substantially the same. In this case rotor speed is limited to 20,000 rpm or about 40,000 *g* because of motor speed limitations.

CHARACTERISTICS OF PLASTIC CHANNELS

One of the attractive features of the "continuous-ring" design is that only a single plastic piece needs to be fabricated. However, the inside wall of the rotor bowl must also be carefully machined. This metal analytical wall could pose some restrictions for certain biological systems. A strong feature of both designs is that the plastic rings are, in a sense, self-sealing; the "O"-rings used in the "continuous-ring" design of Fig. 2 are merely a backup. The fact that both rotors are filled with pressure-equalizing liquid greatly simplifies the sealing of the channel either to the side of the bowl or to another plastic ring, since the pressure difference between the liquid inside the channel and the liquid inside the bowl is negligible.

Special features of the "floating" channel design are that a single central hub is required for the assembly, and that the outer ring is easily removed and replaced. This greatly simplifies cleaning and replacement when channels of different thicknesses or conformations are required. In this replaceable channel design, the SFFF channel is conveniently machined into the outer ring. If desired the channel can be placed on the inner hub, but this limits the system to a single channel conformation unless the whole central hub is replaced.

An especially advantageous aspect of the "floating" channel design (Figs. 3 and 5) is that the easily replaceable outer rings can be constructed with different channel conformations. For example, the optimum aspect ratio of the channel has not yet been established, and studies are underway to construct a series of Noryl channel rings to study this effect.

EXPERIMENTAL

The new plastic channel designs were tested and used in newly developed SFFF instrumentation consisting of a modified Model L5-50B ultracentrifuge (Beckman Instruments, Fullerton, CA, U.S.A.) and a modified Sorvall Model RC-5 Superspeed centrifuge (DuPont Analytical Instruments and Biomedical Products Division, Wilmington, DE, U.S.A.). Each unit was equipped with an improved design of a high-speed, water-cooled, rotating face-seal (see Fig. 7), a DuPont Model 850 microprocessor-controlled solvent-metering system, a Varian "Vari-chrom" UV spectrophotometric detector, a Valco remote air-actuated sampling valve, and a Digital Equipment Corporation MINC-023 computer. The general arrangement of our SFFF apparatus is illustrated by the schematic in Fig. 6. The accessory equipment and the computer software are similar to that previously reported[6]. General procedures for operating this equipment were previously discussed[3].

Biological samples were purchased from Sigma (St. Louis, MO, U.S.A.) or Bethesda Research Labs. (Rockville, MD, U.S.A.). Other experimental samples were obtained from other DuPont laboratories.

A schematic of the rotating face-seal needed to permit liquid to be delivered in and out of the rotating channel is shown in Fig. 7. This unit consists of rotating soft seal mated by spring-loading to a carefully finished stationary seal plate of the form previously described[6]. The soft seal member is driven by a shaft connecting to the rotor bowl cap. This shaft consists of a hollow, flexible nylon torque-tube that contains narrow-bore tubing that connects the mobile phase inlet and the detector to the channel inlet and outlets, respectively (see Fig. 3). The spindle containing the torque-tube shaft assembly rotates inside an oil-fed bronze bearing. The flexible drive-

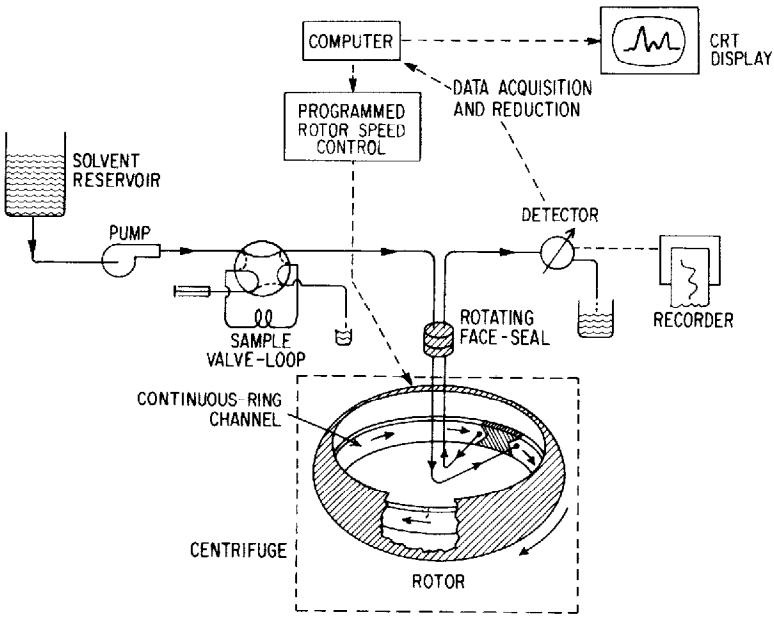

Fig. 6. Schematic of SFFF equipment.

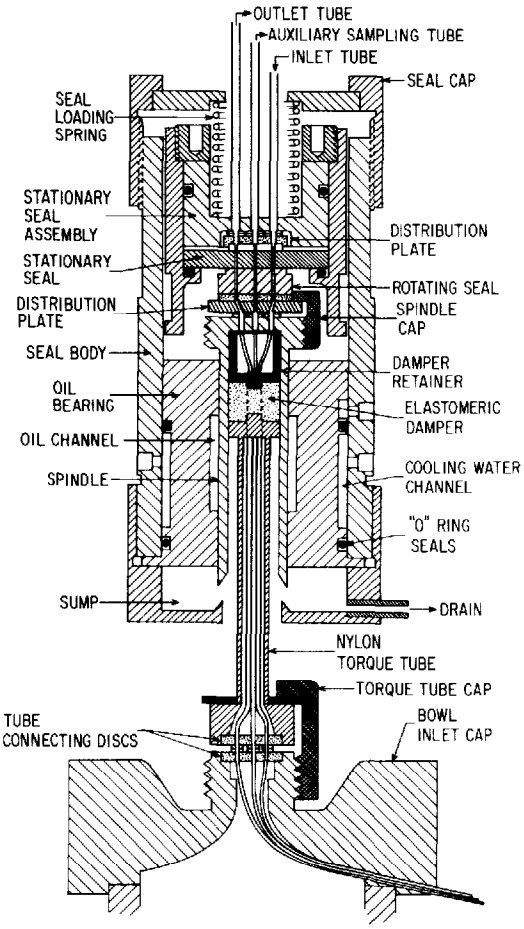

Fig. 7. Rotating face-seal assembly.

shaft[13] and connecting rubber damping system effectively decouple the face-seal from vibrations that might arise from the rotor–motor assembly. This rotating face-seal has worked at 32,000 r.p.m. continuously for months without mobile phase leaks or other problems. Under such use, wear has been minimal so that seal members have survived at least nine months of daily use without need for replacement.

The procedure for obtaining SFFF fractograms with this equipment generally follows those previously described[3,6,7]. Samples are injected into the channel under the initial force field by slowly sweeping the loop of the sampling valve with approximately a three-fold excess of mobile phase liquid. As a typical example, the contents of a 250-μl sample loop might be displaced into the channel by pumping at 0.5 ml/min for 1.5 min —while the channel is operated at (or sometimes above) the initial operating speed for the separation. At this point mobile phase flow is stopped, and the sample is relaxed or equilibrated for a time period dependent on particle mass (theory and operation of the sampling and relaxation step will be described in a future publication). Mobile phase flow-rate is then restored to the value needed for the

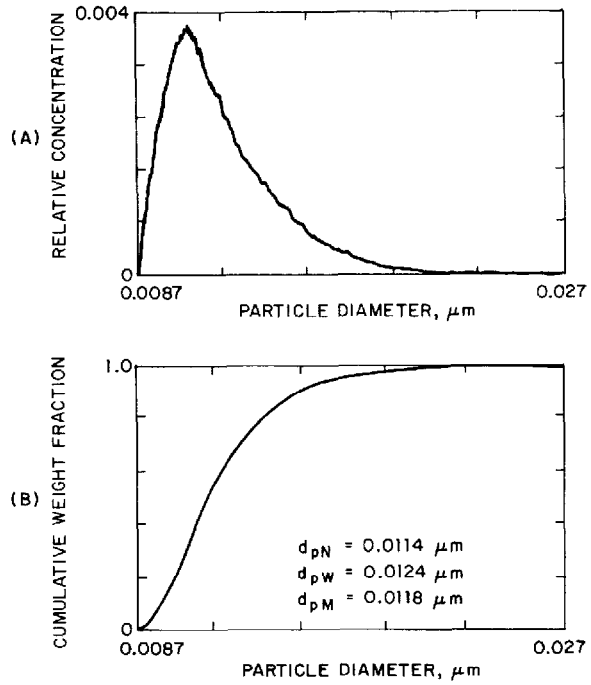

Fig. 8. Aged Ludox-SM colloidal silica. (A) Differential plot of relative concentration *vs.* particle diameter. (B) Cumulative weight fraction *vs.* particle diameter plot; "floating" channel, 0.21 mm; mobile phase, 0.001 *M* NH_4OH; flow-rate 1.0 ml/min; initial rotor speed, 30,000 r.p.m.; delay before exponential decay, 12.0 min; exponential decay time constant τ = 8.0 min; detector, turbidimetric, 230 nm; sample, 250 μl of 1%. d_{pN} = number-average, d_{pW} = weight-average and d_{pM} = mean particle diameters, respectively.

separation, and, when used, the time-delay exponential decay program[7] is also initiated.

RESULTS

The availability of higher force fields permits the characterization of samples not previously accessible to SFFF analysis. For example, measurement of the particle size distribution of very small inorganic colloids now is possible. The differential and cumulative particle size distributions of a Ludox®*-SM colloidal silica sample that had been aged for 6–7 years are shown in Figs. 8A and 8B, respectively. This sample was fractionated in a 0.2 mm "floating" channel using time-delayed exponential SFFF (TDE-SFFF) with an initial rotor speed of 30,000 r.p.m. The weight-average particle diameter of this aged sample was 12.4 nm (0.0124 μm) which is somewhat larger than the nominal size of 8–9 nm seen by transmission electron microscopy when this sample was originally prepared and normally expected for freshly manufactured Ludox-SM colloidal silica. However, this larger size is in keeping with growth of silica colloids in this size range with aging, because of dissolution of smaller

* ® Trademark for DuPont's colloidal silica.

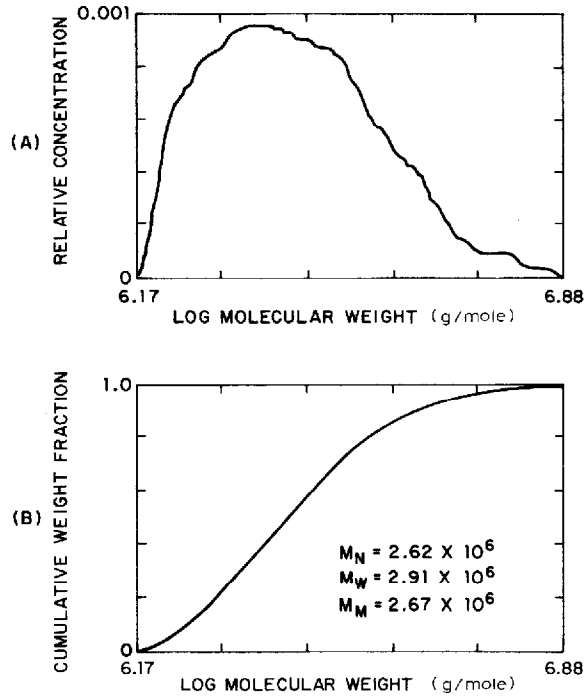

Fig. 9. Polyacrylamide (Aldrich). (A) Differential plot of relative concentration *vs.* log molecular weight. (B) Cumulative weight fraction *vs.* log molecular weight plot; "floating" channel, 0.4 min; mobile phase, 0.001 *M* NH_4OH; flow-rate 1.0 ml/min; initial rotor speed, 32,000 r.p.m.; exponential decay/decay constant, τ = 16.0 min; detector, UV, 200 nm, 0.1 a.u.f.s.; sample, 250 μl, 0.5 mg/ml. M_N = number-average, M_W = weight-average and M_M = mean molecular weights, respectively.

particles, and re-distribution of this silica onto larger particles[14]. It is interesting to note that a 12-nm silica colloidal particle of a density $\varrho_s = 2.2$ has a molecular weight of about $4.4 \cdot 10^5$ (ref. 15). Thus, SFFF appears to be a useful method for characterizing colloids and observing small changes in sizes that can be induced in these materials.

Water-soluble polymers of a molecular weight that are analyzed only with great difficulty by size-exclusion chromatography can be readily characterized by SFFF at high force fields. Fig. 9 shows the relative and cumulative molecular weight distribution plots of a polyacrylamide sample with a reported molecular weight of about $5 \cdot 10^6$. Detection in this case was by ultraviolet absorption at 200 nm. Characterization of this sample by TDE-SFFF indicated number- and weight-average molecular weights of $2.6 \cdot 10^6$ and $2.9 \cdot 10^6$, respectively. Molecular weight distribution characterization of water-soluble polymers such as this with molecular weights higher than *ca.* $1 \cdot 10^6$ is very difficult, if not impossible, by existing methods such as size exclusion chromatography[16].

The availability of higher force fields has significantly enlarged the applicability of SFFF for characterizing macromolecular systems of biological interest. For example, Fig. 10 shows the constant-field fractogram of a plasmid pMB 9 sample having a molecular weight of $3.5 \cdot 10^6$. This separation was carried out in the "con-

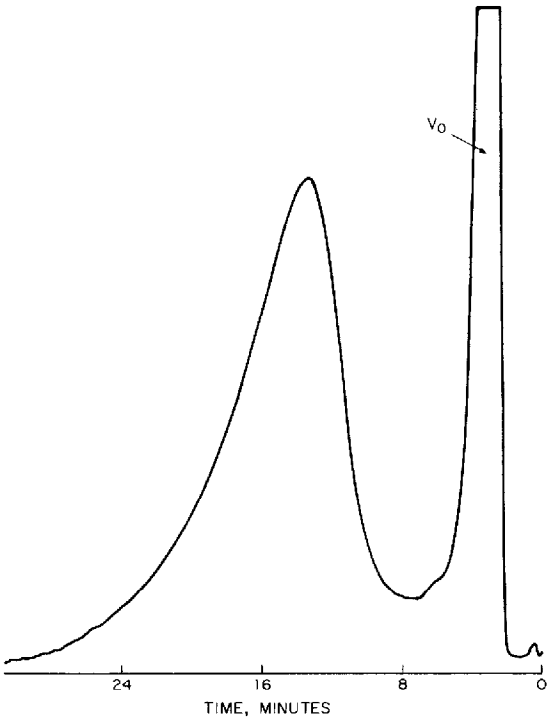

Fig. 10. SFFF fractogram of plasmid pMB 9 (molecular weight $3.5 \cdot 10^6$). "Continuous-ring" channel; 0.25 min; mobile phase, 0.001 *M* Tris buffer, 0.001 *M* NaCl; flow-rate 1.0 ml/min; rotor speed, 20,000 r.p.m. (constant); detector, UV, 260 nm, 0.1 a.u.f.s.; sample, 250 μl, 5 μg.

tinuous-ring" channel using a constant rotor speed of 20,000 r.p.m. The volume (or standard deviation) of the plasmid peak confirms that this is probably a single component of uniform mass and density.

Still lower-molecular-weight components may be fractionated by high force field SFFF, as illustrated in Fig. 11 for fD viral DNA (Miles Labs., Elkhart, IN, U.S.A.; Cat. No. 23-349-1, Lot No. 10). This single-stranded, circular macromolecule was well retained at 30,000 r.p.m. (constant field) with the peak corresponding to a molecular weight of $1.71 \cdot 10^6$, as shown in the cumulative molecular weight plot in Fig. 13. This result compares closely with the reported value (about $1.7 \cdot 10^6$) for this material[17]. The results in Fig. 11B also confirm the narrow molecular weight distribution of this DNA, since its polydispersity (weight-average molecular weight/number-average molecular weight) is essentially unity ($d = 1.018$), within the experimental certainty of SFFF band broadening.

Interestingly, narrow molecular weight distributions are not always observed in the SFFF of DNA preparations. For example, Fig. 12 shows the fractogram for a λ-viral DNA (Miles Labs., Cat. No. 23-351, Lot No. 10). While the calculated molecular weight by SFFF ($33.4 \cdot 10^6$) corresponds closely to that reported for this double-standed DNA[17], the broad peak and relatively large polydispersity ($d = 1.25$) suggest that this DNA sample contains material with a range of molecular weights, or alternatively, components with a range of density distributions (see eqns. 1 and 2). Evidence

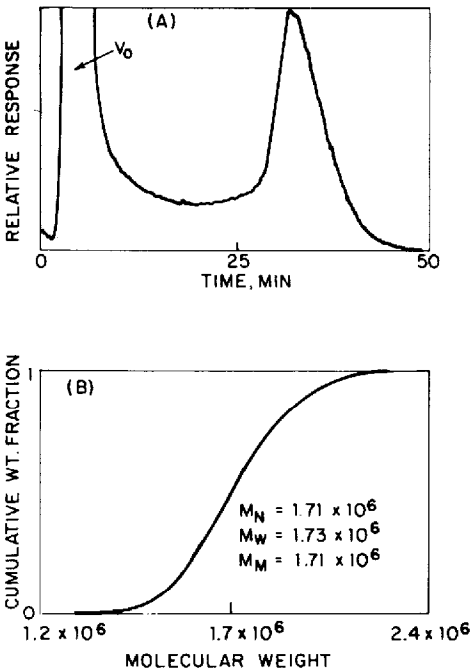

Fig. 11. TDE-SFFF of fD viral DNA. (A) Fractogram. (B) Cumulative molecular weight distribution. Channel, 41.6 × 2.54 × 0.022 cm; mobile phase, 0.05 *M* Tris, 0.1 *M* NaCl, pH 7.5; rotor speed, 30,000 r.p.m. (constant); flow-rate, 0.50 ml/min; sample, 25 μl of 10 units/ml; detector, UV, 260 nm. M_N = number-average, M_W = weight-average and M_M = mean molecular weights, respectively.

for this conclusion is largely based on the facts that the elution volume encompassed by this λ-DNA peak is much larger than that for the fD viral DNA in Fig. 11 (polydispersity *ca.* 1.0), and also much larger than that of the much larger molecular weight, narrow particle size polystyrene latex standards[7]. Preliminary experiments suggest that the relatively broad λ-viral DNA peak is not a function of mass transfer-band broadening. However, additional studies are required to define the reason for this broad peak.

The small peak at about 8 min in Fig. 12 represents a decomposition product of λ-viral DNA which increased with time. This impurity band was not noted when the sample was freshly prepared.

The approximate lower molecular weight limit of our present SFFF equipment for macromolecules of biological interest is illustrated in Fig. 13. Here the fractogram (Fig. 13A) and the differential mol. wt. plot (Fig. 13B) are shown for the protein, equine fibrinogen, reported to have a molecular weight of 501,000 (ref. 17). In this case a thicker (0.031 cm) channel was utilized so that a significant retention would occur, that is, retention ratio $R = V_R/V_0 = 0.37$. A one-hour relaxation period was required for this fractionation. As noted in Fig. 13B, the calculated mean molecular weight of this material by SFFF is $4.95 \cdot 10^5$. The measurable polydispersity (ratio of weight- to number-average molecular weight of $d = 1.036$) is illustrative of the low level of instrumental band broadening observed in SFFF, even with relatively thick channels. This situation makes possible the accurate determination of sample mass

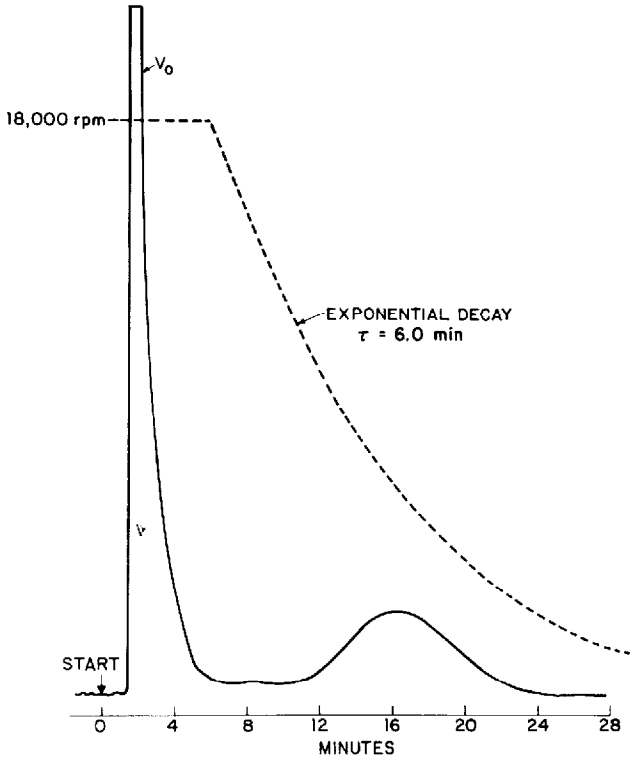

Fig. 12. TDE-SFFF fractogram of λ-viral DNA. Split-ring channel, 57 × 2.54 × 0.0254 cm; mobile phase, 0.001 *M* Tris, 0.001 *M* NaCl, pH 7.6; rotor speed, 18,000 r.p.m. initial; exponential delay-decay constant, 6.0 min; flow-rate, 2.0 ml/min; sample, 50 μl; detector, UV, 260 nm.

without complicated (and inaccurate) corrections for band broadening effects that are required in other mass-defining separation methods such as size-exclusion chromatography[7].

For the separation in Fig. 13, use of an isopropanol-modified mobile phase was required to eliminate sorption of this protein to the polyphenylene oxide channel surface, presumably by hydrophobic effects extensively utilized in reversed-phase liquid chromatography for a wide variety of lower-molecular-weight proteins and peptides. More consistent means of eliminating such sorption are being investigated.

CONCLUSIONS

SFFF equipment has been designed and fabricated which significantly increases molecular weight capability range. Newly designed, water-cooled, rotating face seals and unique plastic rotors containing SFFF channels can be operated at rotor speeds of up to 32,000 r.p.m. (*ca.* 100,000 *g*). Channels of different conformation may be conveniently inserted for particular separation goals or removed to facilitate cleaning.

Two new designs for SFFF channels have been developed for high force-field SFFF operation. One approach involves a "continuous-ring" of plastic containing

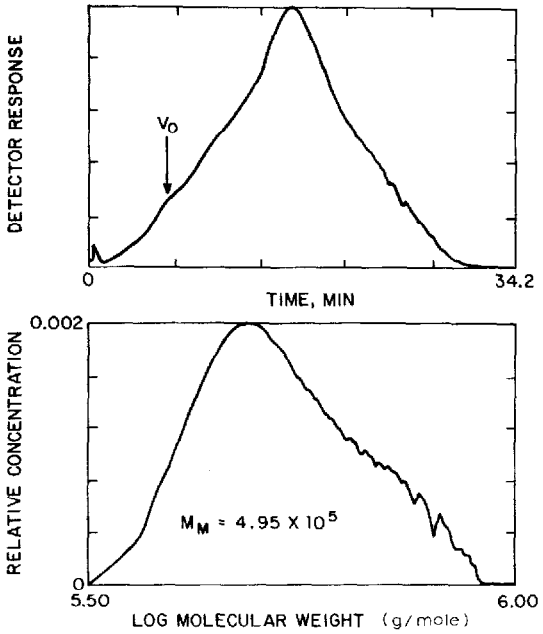

Fig. 13. Fibrinogen (equine, molecular weight 501,000). (A) Fractogram, detector response *vs.* time.) (B) Relative concentration *vs.* log mol. wt. plot; "floating" channel, 0.31 mm; mobile phase, 25% isopropanol in water; flow-rate, 0.50 ml/min; rotor speed, 32,000 r.p.m. (constant), detector, UV, 280 nm; sample, 500 μl, 1.1 mg/ml.

the channel, which is inserted and sealed by compressive fit to the inside polished surface of a metal rotor bowl. Because of inherent elasticity, this plastic ring can enlarge with the bowl as the force field is increased, thus maintaining the integrity of the channel at high force fields. A salient feature of this design is that the rotor assembly is filled with liquid to minimize density differences between the surrounding liquid and the plastic ring and between the surrounding liquid and the liquid in the separating channel, greatly reducing stresses on the plastic and the channel itself.

A second often preferred "floating" channel approach also involves a plastic unit consisting of a central hub typically made of a higher density plastic fitted with an outer ring of lower density plastic. This outer ring containing a carefully machined SFFF channel is shrink-fitted to the central hub to ensure a liquid-tight seal at zero force field. A key feature of this approach is that the total rotor assembly is mounted within a metal rotor bowl and allowed to "float" free of the rotor wall in a liquid of specified density. This unique construction allows liquid to totally surround the hub-ring assembly, essentially equalizing stresses on the plastic parts at high force fields. In addition, pressure differences between the surrounding liquid and the fluid in the channel are very low, making channel leaks unlikely.

Both new plastic rotor designs allow a wide range of mobile phase densities to be used throughout the available force-field range, to permit simultaneous particle density and particle size measurements on a wide variety of particles of both industrial and biochemical interest.

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