Journal of Chromatography, 461 (1989) 73–87
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1481

APPLICATION OF AN ASYMMETRICAL FLOW FIELD-FLOW FRACTIONATION CHANNEL TO THE SEPARATION AND CHARACTERIZATION OF PROTEINS, PLASMIDS, PLASMID FRAGMENTS, POLYSACCHARIDES AND UNICELLULAR ALGAE

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SUMMARY

The asymmetrical flow field-flow fractionation channel has been improved by introduction of a different sample loading method, downstream central injection, which reduces sample relaxation and focusing time considerably and allows the concentration of material in the channel, thereby enabling the loading of very large sample volumes. The performance of the channel was demonstrated by the separation of a protein from its dimer, retention of nucleic acids, *i.e.*, plasmids and enzymatically cleaved plasmids, hyaluronate and unicellular algae.

INTRODUCTION

Flow field-flow fractionation (flow FFF) is the most universally applicable method among the various modes of FFF techniques[1]. Like all other FFF modes, it is usually performed in a thin, ribbon-like, open channel, through which the flow of a liquid carrier is maintained. When some external force field is applied perpendicular to the channel walls, it may interact with sample molecules or particles and drive them to one of the walls, the accumulation wall, where they will accumulate at some defined distance from that wall. Due to the parabolic velocity profile between the channel walls, samples which are present at different distances from the accumulation wall will obtain different velocities when they migrate with the carrier stream down the channel. This differential migration causes the separation.

In flow FFF the force field is a secondary flow of carrier (cross-flow) through the channel walls, which therefore have to be porous. The universality of the method stems from the fact that the cross-flow interacts with any sample molecule or particle in the channel. One limiting factor, however, is the pore size of the semipermeable membrane used as the accumulation wall. This membrane must be permeable to the carrier but not to the sample, and that sets a lower limit for the molecular weights of samples that can be retained in the channels.

Flow FFF has already been shown to separate macromolecular and particulate water-soluble materials of widely different natures, such as proteins[2], viruses[3],

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polystyrene sulphonates[4] and silica sols[5]. Those separations were, however, quite time-consuming, often requiring many hours.

Recent interest in high-speed separations has led to much shorter elution times through the optimization of the flow-rates[6]. The elution times for sulphonated polystyrenes[6] were much reduced and the modernized techniques were used for size fractionation of humic acids and fulvic acids[7]. Most striking has been the very rapid particle separations obtained by flow/steric FFF of polystyrene latex beads in the size range from 2 to 50 μm in diameter[8,9].

The results presented in this paper were obtained in an "asymmetrical" flow FFF channel[10] which was developed in order to simplify the construction of flow FFF channels and to facilitate studies of their properties. This is possible partly because the asymmetrical channel permits the use of a glass wall which makes the channel contents visible. The theory of the operation of the channel was developed[10] and it was shown that under certain conditions the asymmetrical channel can be operated much like a normal "symmetrical" channel but that sample introduction tends to be more cumbersome, requiring a focusing and relaxation procedure that became very time-consuming for high-molecular-weight samples.

In the present study, the asymmetrical channel has been modified by moving the sample loading point from the inlet end to some point downstream and central in the channel. This downstream central injection serves to decrease the sample focusing and relaxation time, but it also permits the loading of much larger sample volumes. In the previous paper on the asymmetrical channel[10], actual separation was demonstrated only for a mixture of three proteins. In the present paper, results are given for a broader range of sample types. The channel was operated both in normal FFF mode and in steric FFF mode. The latter permits the separation of particles, taking advantage of an hyperlayer effect, which originates in hydrodynamical lift forces acting on the particles[8,9,11].

The performance of the modified channel design will be illustrated by its application to the rapid and efficient elution of a protein and its dimer, retention of plasmids, separation of plasmid fragments, obtained after cleavage by restriction enzymes, size fractionation of an high-molecular-weight polysaccharide (sodium hyaluronate) and size fractionation of unicellular fresh-water algae.

EXPERIMENTAL

The flow FFF channel was of the asymmetrical design recently described[10], which has a porous frit mounted in a Lucite block. The frit is covered in sequence by a semipermeable membrane, a PTFE (polytetrafluoroethylene) spacer, a glass plate and a Lucite block. Bolts are used to clamp the assembly together. Two porous frit (porous ceramics P-6-C; Coors Ceramics, Golden, CO, U.S.A.) mountings were a generous gift from Dr. J. C. Giddings, at the Department of Chemistry at the University of Utah, Salt Lake City. The semipermeable membranes were a cellulosic material (Diaflo Ultrafiltration Membrane Type YM; Amicon, Danvers, MA, U.S.A.). A cut, 28.5 cm long and 1.0 cm wide, with triangular ends was made in the PTFE spacers.

Three channels with different membranes, and of different thicknesses, were assembled. They are numbered I, II and III, and have the following characteristics:

I and II had a YM 10 membrane (nominal molecular weight cut-off of 10 000 for globular molecules), and III had a YM 30 membrane (cut-off at 30 000). Channel I had a nominal spacer thickness of 0.032 cm, giving a nominal volume of 0.86 ml, II had a nominal spacer thickness of 0.030 cm and a nominal volume of 0.81 ml and III had a nominal spacer thickness of 0.050 cm and a volume of 1.4 ml. The void volume of channels I and II were determined by injection of copper sulphate as described earlier[10], and was found to be 0.86 and 0.79 ml, respectively. Inlet and outlet holes in the glass plate (made of float-glass) were positioned at a distance of 28.50 cm from each other and fitted with PTFE tubing, as described earlier[10]. The inlet tubing was 1 mm I.D., and the outlet tubing was 0.3 mm I.D. and 8 cm long. The channels were positioned vertically in order to avoid gravitational sedimentation from contributing to the retention of particles.

Sample loading was made through a third hole, positioned 4.0 cm downstream from the inlet hole. It was fitted with PTFE tubing (8 cm × 0.3 mm I.D.) and connected to a Model 7125 sample injection valve (Rheodyne, Cotati, CA, U.S.A.) coupled to an LKB 2150 HPLC pump (Pharmacia LKB Biotechnology, Bromma, Sweden). Sample loops were from 10 to 500 μl in volume. The total hold-up volume between the sample loop and the channel was about 35 μl. The carrier inlet flow was delivered from a Beckman 114M Solvent Delivery System or an Altex 110A Pump (both from Beckman Instruments, Berkeley, CA, U.S.A.). Fluorometric detection of the eluate was performed on-line by a Schoeffel FS 970 L.C. Fluorometer (Kratos Analytical, Ramsey, NJ, U.S.A.). It was equipped with a xenon lamp FSA 170 and a xenon lamp power supply FSA 190 (Kratos Analytical), which were kindly loaned from Pharmacia Therapeutics (Uppsala, Sweden). Absorptiometric detection was performed by a SpectroMonitor III spectrophotometric detector (LDC, Riviera Beach, FL, U.S.A.), set at 280 nm for proteins, 260 nm for plasmids and 420 nm for the algae. The main detector response from the algae is believed to be due to light scattering by the algal cells. The three-way and four-way manual switching valves (see Fig. 1) were of Types SRV-3 and SRV-4 (Pharmacia, Uppsala, Sweden). The needle valve N1 was a Micro Metering valve SS-22RS2 (Whitey, Highland Heights, OH, U.S.A.). The needle valves N2 and N3 were Fine Metering valves SS-SS1 (Nupro, Willoughby, OH, U.S.A.).

The operating procedures followed those given previously[10] with exceptions due to the introduction of the new sample loading procedure. With valves V1 and V3 (Fig. 1) set for the relaxation/focusing mode, sample loading was effected by having pump 2 work at a flow-rate usually of 0.05 ml/min except for large volume samples (>100 μl) when it was 0.25 ml/min. The flow from pump 2 was maintained for a time sufficient to load the complete sample into the channel. This usually required a volume of twice the loop volume and the hold-up volume. Sample volumes greater than 500 μl were obtained by repeated filling of the loop.

Fig. 2 illustrates the flow patterns in the channel.

Human serum albumin (HSA) Fraction V was from Sigma (St. Louis, MO, U.S.A.). The plasmids were kindly supplied by the Department of Pharmaceutical Microbiology (University of Uppsala, Uppsala) and Pharmacia Biotechnology. Dextran and sodium hyaluronate, tagged with fluorescein, were kindly supplied by Pharmacia Ophthalmics (Uppsala, Sweden). They were reported to have weight-average molecular weights of $\approx 150\,000$ and 79 500 daltons respectively. Samples of

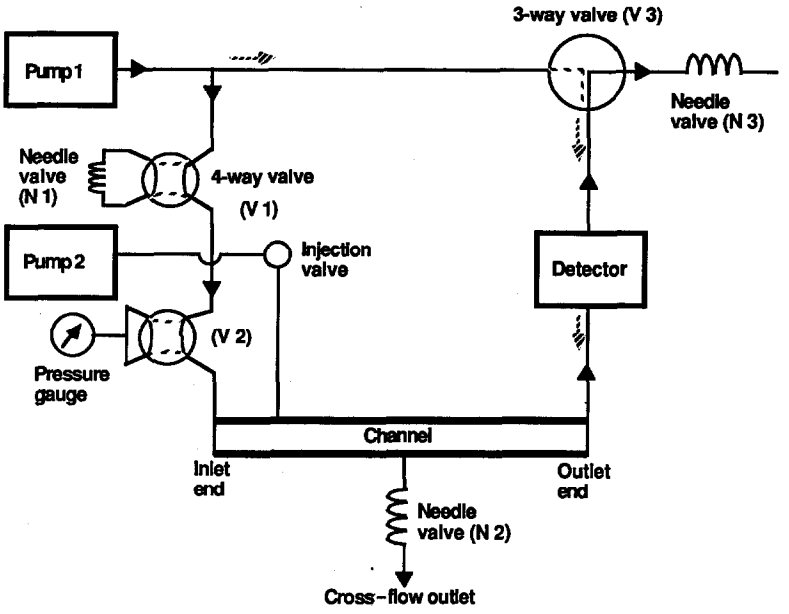


Fig. 1. Schematic drawing of apparatus for flow FFF with asymmetrical channel. The flow directions indicated are used for elution. The dashed lines and arrows show the flow directions during sample loading, relaxation and focusing. Simultaneous switching of valves V1 and V3 is used to shift between focusing/relaxation and elution mode. By switching valve V2 the pressure in the channel can be read. The needle valve N1 is used to adjust the focusing point. Adjustment of N2 and N3 determines the ratio between the cross-flow-rate and channel outlet flow-rate.

various species of algae were kindly donated by the Department of Limnology (University of Uppsala, Uppsala) and were suspended in a culture medium or resuspended in the carrier. The carrier was a Tris–HNO_3 buffer (pH 7.4), ionic strength 0.1, containing 0.02% sodium azide with the addition of 1 m*M* EDTA for the plasmid experiments.

RESULTS AND DISCUSSION

The data treatment follows that of ref. 10 and the following symbols are used: z' = focusing point (distance from inlet end), $\dot{V}_c'$ = cross-flow-rate during relaxation, $\dot{V}_c''$ = cross-flow-rate during elution, $\dot{V}_{out}$ = flow-rate at channel outlet end, t^0 = void time and t_R = retention time.

Downstream central sample injection

The previous study[10] of the asymmetrical channel showed that sample relaxation is done best by a so-called opposing-flow relaxation which involves focusing of the sample to a point some distance (a few cm) down the channel. To obtain a narrow starting zone of the sample, it is then required that it migrates from the sample loading point at the inlet end down to the focusing point. It was shown

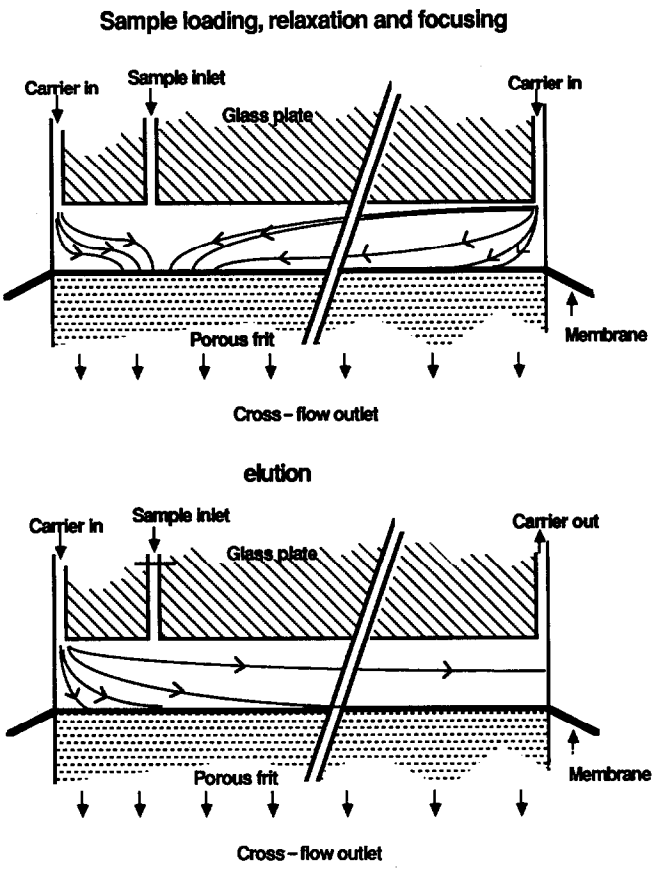


Fig. 2. Illustration of the function of the asymmetrical flow FFF channel in sample loading, relaxation/focusing mode and in elution mode. Streamlines are only approximate.

theoretically[10] that this would require an inconveniently long time for high-molecular-weight samples having a low diffusion coefficient and therefore a low migration velocity. The obvious way to reduce focusing time is to load the sample directly at the predetermined focusing point. This was done by inserting a sample loading tube into the glass plate a few cm downstream from the channel inlet end and on the centre line of the channel. Sample loading is then accomplished while the flowstreams in the channel are in the opposing-flow relaxation mode. The focusing point, regulated by the relative magnitude of the two opposing flowstreams, is preferably located a few mm downstream from the sample loading point. This is done in order to prevent the sample-loading flowstream from flushing away sample material, already loaded, from the focusing point. Experiments with a coloured sample (Blue dextran) showed that the visible zone length of the starting zone became about 5 mm. The zone length will depend on the magnitude of the sample loading flow-rate relative to the opposing flow-rates. The loaded sample exhibited a more or less elliptical zone shape. With this new sample loading technique, the total time for loading, focusing and relaxation of

small-volume samples can be kept below 2.5 min, even for high-molecular-weight samples, as demonstrated in the following paragraphs.

The downstream central injection has two more advantages.

(1) It will allow the loading of large sample volumes, because they will be compressed in the focusing procedure. Very large sample volumes may require long loading times because the sample-loading flow-rate must be sufficiently low in relation to the opposing flows in order to keep the starting zone small. As much as 5 ml of a sample have been loaded in a channel having a void volume of only 0.8 ml.

(2) The other advantage is that the sample is loaded on the central line of the channel and may not reach the edges of the channel, even during the elution. Therefore, the influence of adverse "edge effects"[12] may be decreased.

Separation of protein monomer and dimer

The separation of proteins by flow FFF has been demonstrated a number of times. Early efforts[13,14] were very time-consuming (often several hours), and the resolution was always limited by higher-than-predicted plate heights, H (ref. 14). When flow optimization similar to that in ref. 6 is used in order to obtain rapid elution, in combination with high efficiency, the plate height still appears high for the three proteins separated[10]. The flow optimization is based on the concomitant increase in longitudinal flow velocity and cross-flow velocity[6] so that the increased longitudinal velocity is balanced by an increased retention level. Because the non-equilibrium plate height decreases with the square of the channel thickness, w^2, at constant retention degree[6], it was thought that the 0.03-cm thick channel used in this work would lead to improved plate heights, as compared to the 0.05-cm thick channel used in the previous work[10].

The fractogram in Fig. 3 shows the elution of a sample of HSA under flow conditions that give rapid elution and very high retention, corresponding to 29 void times for the main peak, which is the albumin monomer. The second peak, well resolved from the monomer, is thought to be the dimer of albumin. This fractogram also appears to represent one of the most efficient elutions by flow FFF; the number of plates, calculated for the monomer peak, is 518, which corresponds to an average plate height, $\bar{H}$, of 0.47 mm. In some other, more optimized experiments, even higher plate numbers, 700, were obtained. The longitudinal velocity decreases linearly from the inlet end to the outlet end[10] resulting in a decreased local plate height. Still, it can be shown that the observed plate height is very much above the expected non-equilibrium plate height, even if this is calculated for the longitudinal velocity valid at the inlet.

It is expected that conditions similar to that of Fig. 3 can be used to separate and detect higher aggregates of proteins, perhaps using flow programming to optimize the elution time of the higher aggregates.

Retention of plasmids and separation of plasmid fragments

In working with plasmids and other nucleic acids there is often a need to fractionate the material according to size. The dominating fractionating procedure is agarose gel electrophoresis, involving, first, the electrophoretic separation in the gel, and then the extraction of the separated zones from the gel by, *e.g.*, isotachophoresis[15]. Such procedures, although requiring relatively simple equipment, tend to be rather time-consuming. Separation would be much faster with flow FFF, which has the

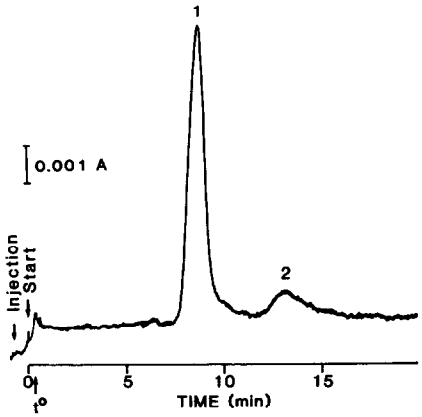
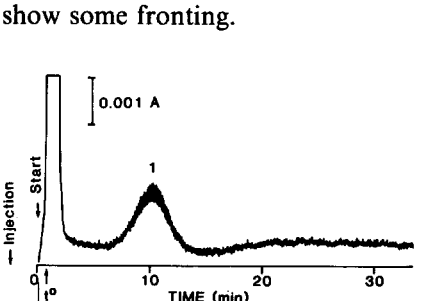


Fig. 3. Separation of the monomer and dimer of HSA (channel II). Peaks: 1 = monomer; 2 = dimer. Sample: 10 mg/ml HSA, $\approx$1 μl. Relaxation/focusing: focusing point (distance from inlet; z' = 4.1 cm, $\dot{V}_c'$ = 4 ml/min; elution, $\dot{V}_c$ = 5.37 ml/min, $\dot{V}_{out}$ = 0.72 ml/min, t^0 = 0.30 min. Observed diffusion coefficient for peak 1 is $5.8 \cdot 10^{-7}$ cm^2/s.

further advantage that the material can be kept in any suitable aqueous solution and that the procedure is easily handled or automated. Another advantage is that simply by manipulating the flow-rates one can apply the method to materials over a very broad size range. The resolution of flow FFF may not be as high as that of electrophoresis, and the method seems therefore most suitable for the fractionation of material showing relatively large differences in molecular weight. Separations of plasmids and other DNA material have been demonstrated by Kirkland and co-workers[16] by sedimentation FFF, which is capable of higher separation selectivity than flow FFF. However, the sedimentation FFF apparatus is much more complex and expensive.

Figs. 4 and 5 demonstrate the retention of two different plasmids. The elution time of each of them was easily adjusted by chosing flow-rates based on an approximate estimation of their diffusion coefficients. The retention ratios were 0.077 and 0.050, respectively. When higher retention levels were chosen, the peaks tended to show some fronting.



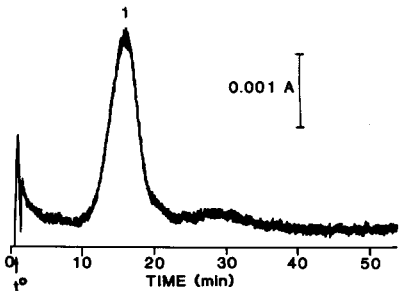


Fig. 4. Isolation of the plasmid pGL 101 (2390 base pairs) by flow FFF (channel II). Sample: 0.5 μg/μl pGL 101, 4 μl. Relaxation/focusing: z' = 4.1 cm, $\dot{V}_c'$ = 4 ml/min. Elution: $\dot{V}_c$ = 0.27 ml/min, $\dot{V}_{out}$ = 0.75 ml/min, t^0 = 0.78 min.

Fig. 5. Isolation of the plasmid pBR 322 (4360 base pairs) by flow FFF (channel II). Sample: 0.5 μg/μl pBR 322, 2 μl. Other conditions as in Fig. 4.

One obvious application of flow FFF would be the isolation of, *e.g.*, small fragments from the large fragments that are obtained upon cleavage of plasmids with restriction enzymes. Such experiments are illustrated in Fig. 6. A plasmid, pTL 830, was cleaved by three different enzymes or enzyme combinations in order to give fragments of the sizes given in the Figure legend. The elution curves correspond very well with predictions regarding both the position and the relative area of the peaks obtained from the two fragments. The resolution of the two peaks increases with increasing difference in the number of base pairs and the peak-area ratio changes approximately in proportion to the ratio of the number of base pairs in each fragment. The poor shape of the large fragments may be due to the very high retention of these peaks, in one case corresponding to 65 void times. Flow programming[6] can easily be used to speed up the elution of highly retained peaks.

The large response sometimes occurring close to the void time was also seen in blank experiments; it is perhaps caused by pressure pulses. In some cases, such as the fractogram at the bottom right, an extra response close to the void time but not occurring in the blank was obtained; this is possibly caused by some impurity in the sample and requires further study.

Positive identifications of the small fragments were obtained by agarose gel electrophoresis for peaks 1 in the two top fractograms. The third case was not tested. The purity and identity of the large fragment in the top right fractogram was tested by collecting a 5-ml fraction. It was reinjected and eluted under the same conditions as for the parent fractogram. As expected, peak 1 had now disappeared, and the area of the

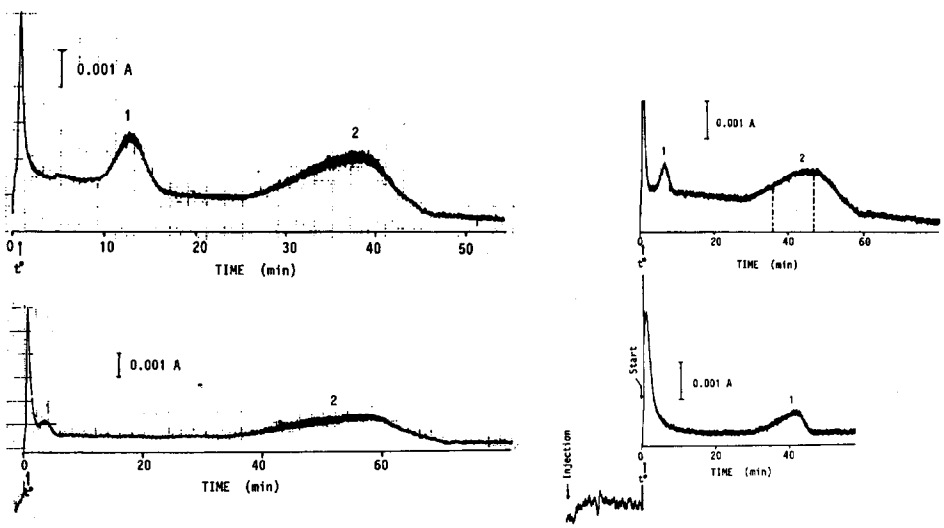


Fig. 6. Fractograms of pTL 830 (5300 base pairs) after cleavage with different restriction enzymes. Reinjection of collected large-volume fraction (channel II). Sample at top and bottom left, and top right: digests of 0.4 μg/μl pTL 830, 4 μl. Relaxation/focusing; $z' = 4.7$ cm, $\dot{V}_c' = 4$ ml/min. Elution; $\dot{V}_c = 0.33$ ml/min, $\dot{V}_{out} = 0.66$ ml/min, $t^0 = 0.85$ min. Left top: cleavage with EcoR1 (1200 base pairs + 4100 base pairs). Left bottom: cleavage with BamH1 and Bg1II (200 base pairs + 5100 base pairs). Right top: cleavage with PstI and Bg1II (700 base pairs + 4600 base pairs). Right bottom: reinjection of a 5-ml fraction, collected from peak 2 of the right top fractogram, as indicated by the dashed lines. Injection flow: 0.25 ml/min for 21 min.

reinjected peak corresponds reasonably well to the area of the fraction collected. This procedure demonstrates perfectly how very large sample volumes can be loaded in the asymmetrical flow FFF channel.

These results show the great potential of the application of flow FFF to the size fractionation of nucleic acids.

Size fractionation of hyaluronate

The study of the size distribution of macromolecules is often an important step in their characterization. The traditional method for this has been size-exclusion chromatography (SEC). However, the pore size of the packing material sets a limit to the upper value for the molecular weight of samples that can be retained in such a column.

With flow FFF one can fractionate, on an analytical scale, macromolecules and particles ranging from molecular weights as low as about 1000 to particle sizes of about 50 μm[1]. Thus, flow FFF can be not only a complement to SEC but can also be used to perform macromolecular fractionations in the high-molecular-weight range where SEC has limited applicability. Sodium hyaluronate is used in eye surgery due to its viscoelastic properties. Its molecular weight can vary greatly, but the material used for medical work usually has an average molecular weight of about $5 \cdot 10^6$ daltons. Fractionation of such material by SEC tends to be very time-consuming due to the high molecular weights which usually require low flow-rates. Also, the elution times tend to vary with sample concentration[17] and it is known that the diffusion coefficient is concentration-dependent[18].

Since natural sodium hyaluronate has a low detection sensitivity in UV absorption and refractive index measurements we had to use sodium hyaluronate tagged with fluorescein for this study. This can be detected at low concentrations by a fluorescence detector. The weight-average molecular weight (MW) of this sample was 79 500 daltons. The properties of the native, and often more high-molecular-weight, sodium hyaluronate may be different from those of the tagged sodium hyaluronate, but the latter may nevertheless serve as a model compound in an initial study of the possibilities and problems in using flow FFF for an high-molecular-weight polysaccharide. Application to native sodium hyaluronate will require the development of more sensitive detection techniques.

Effect of sample amount

Since the parameter that governs retention in flow FFF is the diffusion coefficient of the sample[4], which can be calculated from the elution time[10], we studied the observed diffusion coefficients as a function of the sample amount (Fig. 7). We found that less than 6 μg of hyaluronate must be injected in order to obtain diffusion coefficients that are independent of sample amount. Such low amounts require very sensitive detection, which was provided by the fluorescence detector. With the dextran sample, the limiting sample amount was 2 μg.

Fractionation and reinjection

Hyaluronate is a polydisperse material, exhibiting a broad range of molecular weights. If such a sample is eluted through the flow FFF channel, its contents will be fractionated along the elution time axis, the smallest molecules being eluted first. The

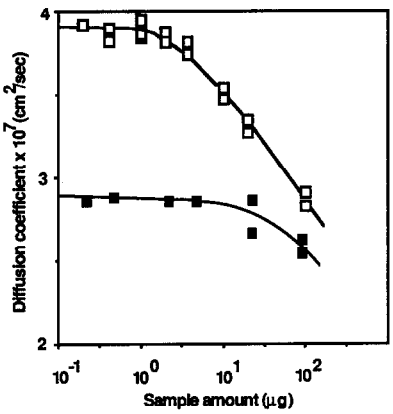


Fig. 7. Sample concentration effects on the observed diffusion coefficient. □ = Fluorescein-tagged dextran, weight-average MW ≈150000 daltons. Relaxation/focusing: $z' = 4.3$ cm, $\dot{V}_c' = 2.5$ ml/min. Elution: $\dot{V}_c = 0.95$ ml/min, $\dot{V}_{out} = 0.53$ ml/min, $t^0 = 0.89$ min, $t_R = 6.9$–8.8 min (channel I). ■ = Fluorescein-tagged sodium hyaluronate, weight-average MW = 79500 daltons. Relaxation/focusing: $z' = 4.2$ cm, $\dot{V}_c' = 4$ ml/min. Elution: $\dot{V}_c = 0.80$ ml/min, $\dot{V}_{out} = 0.44$ ml/min, $t^0 = 0.95$ min, $t_R = 7.8$–9.4 min (channel II).

resulting elution profile will be a more or less broad peak. Peak broadening is also caused by non-equilibrium processes during the elution, and it needs to be shown that the observed peak width is the result of the fractionation process. This can be done by isolating small fractions and reinjecting them into the channel[19]. The elution profiles resulting from the injection of such fractions should then occur at elution times identical to those at which each fraction was collected. Such experiments are summarized in Fig. 8.

The reinjected fractions all occur at elution times that coincide well with the collection time. This shows that a true size fractionation of the hyaluronate has been obtained.

Measurement of the diffusion coefficient

Since the diffusion coefficient is the property of the sample that determines the retention and it may be necessary to compare the results of fractionations obtained

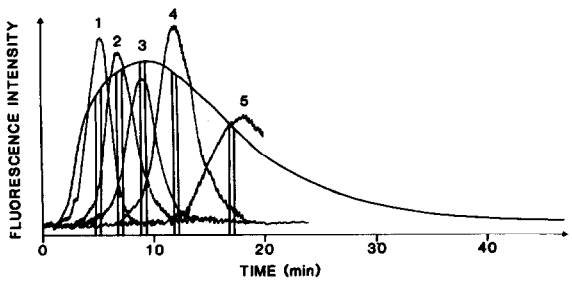


Fig. 8. Fractionation of sodium hyaluronate, followed by reinjection of collected fractions. The broad curve is from the parent sample from which five narrow fractions, 0.25 min wide, were collected (indicated by the vertical lines) and reinjected. The five fractions were analyzed under the same conditions as for the parent sample. Parent sample: hyaluronate tagged with fluorescein (4.5 µg in 98 µl). Relaxation/focusing: $z' = 4.3$ cm, $\dot{V}_c' = 4$ ml/min. Elution: $\dot{V}_c = 0.80$ ml/min, $\dot{V}_{out} = 0.47$ ml/min, $t^0 = 0.89$ min (channel II).

under different flow conditions which may give different retention levels, it may be advantageous to transform the elution time curves into a frequency distribution of diffusion coefficients. Such results are demonstrated in Fig. 9. Each fractogram was digitized and the abscissa was transferred to give diffusion coefficients using the known quantitative relationships between elution time and diffusion coefficient[10]. The ordinate values were multiplied by $\Delta t_R/\Delta D$, where Δt_R is the difference in elution time for consecutive digitized points and ΔD the corresponding difference in diffusion coefficient. Each distribution was normalized so that the area under the curve equalled unity. This transformation ensures that the correct proportionality between the sample mass and the area under the curve is maintained (*cf.*, refs. 7 and 20). One of the curves was obtained with large sample load (92 μg, *i.e.*, above the limit in Fig. 7) and the other two with smaller loads (<2 μg, *i.e.*, below the limit). The distribution curve for the large amount deviates, because the above mentioned limit of sample amount was exceeded. Experiments made with loads lower than 6 μg gave distribution curves that coincided irrespective of the flow conditions in the channel. However, deviations were observed for very high and low retention levels. This shows that conditions can be found under which consistent values of diffusion coefficients can be obtained. These results indicate that flow FFF may be used for size fractionation of relatively high-molecular-weight water-soluble polysaccharides.

Fractionation of unicellular fresh-water algae

The principle of steric FFF can be used to separate particles in the size range 1–100 μm[21,22]. The resolution in steric FFF depends directly on the particle radius because the particles are driven by the cross flow to contact the accumulation wall, and their equilibrium level above the wall is thus determined by their own physical size. Successful separations of human and animal cells, which have sizes in the range mentioned, were demonstrated by the use of sedimentation/steric FFF[23]. Extremely

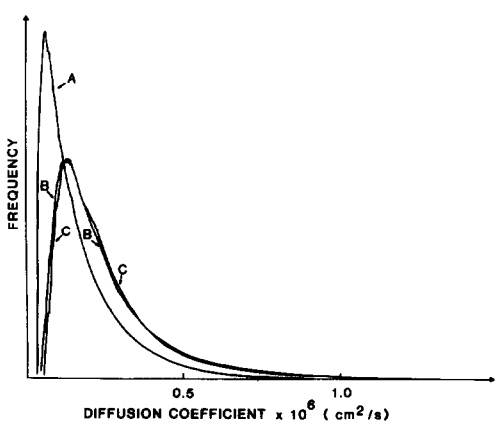


Fig. 9. Distribution of the observed diffusion coefficient of hyaluronate, tagged with fluorescein. Effect of sample amount (channel II). Relaxation/focusing: $z' = 4.3$ cm (B+C) and 4.6 cm (A), $\dot{V}_c' = 4$ ml/min. (A) Sample amount, 92 μg; elution, $\dot{V}_c = 0.82$ ml/min, $\dot{V}_{out} = 0.43$ ml/min, $t^0 = 0.93$ min. (B) Sample amount, 2.2 μg; elution, $\dot{V}_c = 0.80$ ml/min, $\dot{V}_{out} = 0.42$ ml/min, $t^0 = 0.97$ min. (C) Sample amount, 0.92 μg; elution, $\dot{V}_c = 0.55$ ml/min, $\dot{V}_{out} = 0.37$ ml/min, $t^0 = 0.20$ ml/min.

rapid fractionation of polystyrene particles with sedimentation/steric FFF at very high flow-rates, in combination with high field strength, showed[11] that the separation process is influenced by a hydrodynamical lift force which tends to focus the particles at some distance from the accumulation wall which is greater than the particle radius. Such a situation fulfils the requirements for hyperlayer FFF[24] which was also termed focusing FFF[25,26]. The hyperlayer mode is also operational in flow/steric FFF, resulting in very rapid separations of polystyrene beads in the range of 2–50 μm in diameter[8,9].

It is of interest to attempt the application of this principle to the separation of biological cells having sizes in the range mentioned. One such application might be the size fractionation of unicellular fresh-water algae. In limnology one is often faced with the problem of obtaining a size fractionation of natural water samples as a means of indicating the variations in cell size of algae. It is known, *e.g.*, that zooplankton feed on unicellular algae and that different species of zooplankton prefer different species of algae, depending on algal cell size.

A few different species of algae were chosen for study. The experiments followed the principles given earlier for the operation of flow/steric FFF[8,9], with the channel positioned vertically to avoid the influence of gravity on the retention[9]. However, the previous studies were made in the symmetrical flow FFF channel, where the cross-flow velocity is uniform over the channel thickness. In the present work, with the asymmetrical channel, the decreasing cross-flow velocity obtained on going from the accumulation wall to the "upper" wall[10] makes the balance between the presumed lift forces and the cross-flow-induced drift towards the accumulation wall different from that in the symmetrical channel. When the equilibrium distance of the particles from the accumulation wall is greater than 0.06 of the channel thickness, they are in a region of changing cross-flow velocity gradient[10]. This would correspond to a retention ratio [the elution time, t^0, of an unretained component (void time) divided by the elution time of the retained sample, t_R] of >0.35, *i.e.*, very weak retention (corresponding to about <3 void times). It is above this retention ratio that different results are expected in the two types of channels. However, none of the results presented below was obtained under such conditions.

Table I shows the different species of algae used for this study together with their approximate sizes. The size distribution of these algae may be rather broad resulting in relatively wide elution profiles. Moreover, the real cell size may be somewhat different from the value given. The results obtained are rather preliminary, and a more detailed study would require much more work to characterize the original samples with respect to cell shape, size and size distribution.

TABLE I
SPECIES OF UNICELLULAR FRESH-WATER ALGAE STUDIED

Species (and abbreviation)	*Approximate average size (μm)*
Chlorella sp. (Csp)	2.5
Chlorella homosphaera (Ch)	5.3
Haematococcus capensis var. *borealis* (Hcb)	15
Haematococcus droebackiensis (Hd)	21

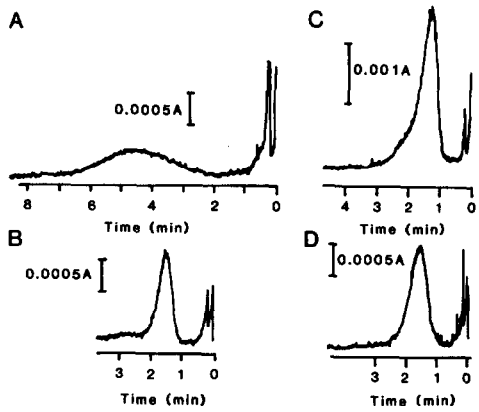


Fig. 10. Fractograms of the four different species of unicellular algae of Table I (channel III). Relaxation/focusing: $z' = 4.3$ cm, time = 3 min, $\dot{V}_c' = 3.0$ ml/min. (A) Csp, $\dot{V}_c = 0.43$ ml/min, $\dot{V}_{out} = 5.06$ ml/min, $t^0 = 0.23$ min;(B) Ch, $\dot{V}_c = 0.29$ ml/min, $\dot{V}_{out} = 7.32$ ml/min, $t^0 = 0.16$ min; (C) Hcb, $\dot{V}_c = 0.29$ ml/min, $\dot{V}_{out} = 6.98$ ml/min, $t^0 = 0.17$ min; (D) Hd, $\dot{V}_c = 0.34$ ml/min, $\dot{V}_{out} = 7.06$ ml/min, $t^0 = 0.17$ min.

Fig. 10A–D show fractograms of the individual samples. The adjustment of retention times for each species was based on the experience gained in the previous study[8,9]. Larger cells would be eluted before smaller cells, and this is the expected elution order in steric FFF. Increases in retention time can be obtained by either increasing the "field strength", *i.e.*, the cross-flow rate, $\dot{V}_c$, or decreasing the longitudinal flow-rate ("channel flow-rate"), as measured at the outlet of the channel, $\dot{V}_{out}$. An increased cross-flow rate increases the retention degree, whereas the main effect of decreasing the channel flow-rate is to decrease the longitudinal carrier velocity. The flow conditions were adjusted so that the elution times of the various samples of algae fell below *ca.* 5 min.

Rather well shaped peaks were obtained from each population of algae. Data in Fig. 10B–D were obtained under similar conditions and the separation of the three species was slight. They all were eluted at approximately the same time, although their sizes range from 5 to 21 μm. However, they were clearly retained, because the void time was only a fraction of a minute under the conditions used. Thus, efforts to separate mixtures of these species failed. Much more detailed studies are needed in order to find the reason for the low resolution. However, the smallest species (Csp) shows a much longer elution time than the others, and when it is mixed with the largest species (Hd) the two populations can be partially fractionated, as shown in Fig. 11. The decrease in elution time of Csp on going from Fig. 10 to Fig. 11 depends on the change in flow-rates. The lower cross-flow rate decreases the retention degree (increases the retention ratio), while the higher channel flow-rate speeds up elution.

The identity of the zones obtained was checked in only one case by optical microscopy. A collected fraction of Hcb contained cells of algae of the same size and shape as that in the original sample, and there is no reason to believe that the other response curves obtained would be caused by anything else than the respective sizes of algae. The fractogram of Fig. 11 was reproduced several times with the same result.

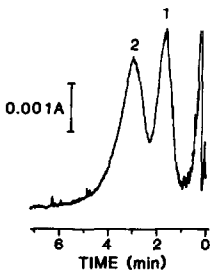


Fig. 11. Fractogram of a mixture of two populations of unicellular algae (channel III). Peaks: 1 = Hd (21 μm); 2 = Csp (2.5 μm). Relaxation/focusing: z' = 4.4 cm, time = 3 min, $\dot{V}_c'$ = 3.0 ml/min. Elution: $\dot{V}_c$ = 0.30 ml/min, $\dot{V}_{out}$ = 7.06 ml/min, t^0 = 0.17 min.

The successful and rapid separation in Fig. 11 gives hope that flow/steric FFF eventually can be developed into a new method of analyzing the size distribution of cells.

ACKNOWLEDGEMENTS

We gratefully acknowledge the help of Drs. R. Bell and L. Bern (Department of Limnology at the University of Uppsala) for the gift of samples of algae and many fruitful discussions. Dr. G. Swedberg (Department of Pharmaceutical Microbiology, University of Uppsala) kindly provided cleaved plasmids and performed the electrophoretic identification. Dr. P. Månsson (Pharmacia Ophtalmics, Uppsala) kindly supplied the dextran and hyaluronate.

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