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Stopless Flow Injection in Asymmetrical Flow Field-Flow Fractionation Using a Frit Inlet

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Stopless flow operation of asymmetrical flow field-flow fractionation (FFF) has been achieved by introducing a hydrodynamic relaxation method using a frit inlet. By using frit inlet injection, a focusing process which has been an essential part of runs at the asymmetrical flow FFF system can be completely avoided. Band broadening of an initial sample zone during hydrodynamic relaxation is discussed with equations related to the ratio of two inlet flow rates. For the successful achievement of particle relaxation and separation, it is necessary to apply a small ratio of sample inlet to frit inlet flow rate. Experimental results are reported for the evaluation of the system efficiency at various levels of hydrodynamic relaxation and for both normal and steric/hyperlayer modes of FFF runs using latex standards. Most importantly, it is shown that a high resolution and a high-speed separation of submicrometer-sized latex mixtures can be accomplished in asymmetrical flow FFF without using the conventional focusing relaxation process.

Flow field-flow fractionation (flow FFF) as a member of the FFF family is a universal separation technique capable of separating and characterizing colloidal particles and macromolecules over a broad molecular weight range.[1−7] In all FFF techniques, separation is carried out in a thin flat channel by the interaction of separation flow with an external force which plays an important role in retaining sample materials in the channel. For the conventional symmetrical flow FFF, a crossflow which moves from the top to the bottom wall of the channel through permeable frits is utilized as a driving force. A separation flow (axial flow) carries sample components to the channel outlet, while crossflow drives them toward the bottom of the channel wall. In the case of asymmetrical flow FFF, an analog to the conventional flow FFF which has been demonstrated to serve as a high-resolution technique for the separation of proteins, DNA, etc., there exists only one permeable wall on the channel bottom.[7−10] The inlet flow in asymmetrical flow FFF is divided into both the channel outlet and the crossflow outlet, and thus the part of the carrier flow which passed through the bottom wall also serves as the crossflow.

Normally, sample materials injected into most FFF systems require a separate relaxation process prior to the separation, during which they are driven to their equilibrium positions near the bottom wall (or accumulation wall) by the action of an external field. For a conventional symmetrical flow FFF channel, the axial flow is stopped for a certain period to allow this relaxation process to occur in order to keep the initial sample band from undue spreading along the separation axis; this is the so-called stop-flow procedure.[2,3] For an asymmetrical flow FFF, sample relaxation is normally achieved by using a focusing process in which two counterdirected flow streams (one from the channel inlet and the other from the channel outlet) converge below the injection point, which is near the channel inlet.[7,8] The focusing action of the two flow streams has been shown to play an important role in reducing the initial bandwidth in addition to achieving a successful sample relaxation. In practice, separation in asymmetrical flow FFF begins by resuming axial flow right after the focusing process is completed.

While the stop-flow and focusing processes are essential in each subtechnique, they are basically cumbersome in the system operation. In addition, they may lead to baseline shifts during the flow conversion and also may lengthen the separation time.[11−13] Earlier publications warn that stoppage of particle migration during the stop-flow process may lead to particle adhesion to the channel wall.[12,13] For these reasons, it is desirable to avoid stop-flow and focusing processes if a suitable relaxation process using stopless flow operation could be incorporated. In the case of a conventional symmetrical flow FFF channel, hydrodynamic relaxation methods were recently introduced by implementing a split inlet or a frit inlet.[13,14] In hydrodynamic relaxation, sample materials are quickly transported to their equilibrium positions by the compressing action of a relatively fast substream of either frit inlet flow or split inlet flow. While these modifications completely remove a stop-flow procedure and reduce the relaxational band broadening which often occurred in stopless flow injection, they require a manipulation of three different inlet flow streams, namely the sample inlet substream, the frit inlet flow or split inlet flow, and the crossflow. Since the sample flow and the frit flow (or split flow) together must exit through the channel outlet in these systems, the

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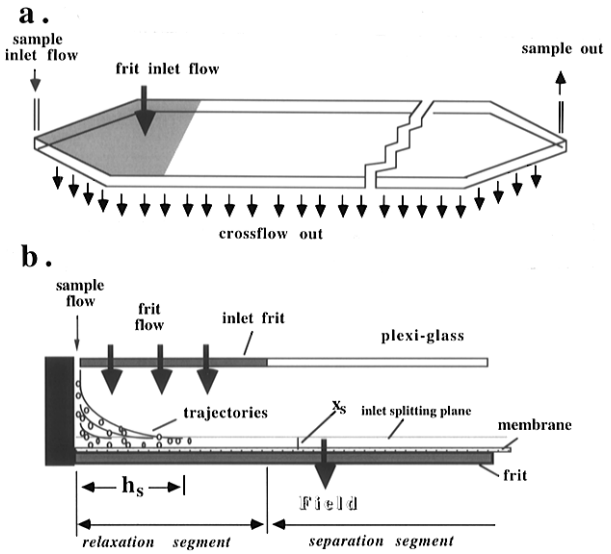

Figure 1. Diagrams showing (a) a permeable frit inlet configuration at the inlet end of the asymmetrical flow FFF channel and (b) hydrodynamic relaxation illustrated from the side of the channel.

resulting high axial flow might result in the dilution of sample materials entering the detector.

While the conventional asymmetrical flow FFF system was demonstrated to be efficient in high-speed separations, sample relaxation primarily relies on the focusing procedure, and one may still encounter the possible problems described above.[10] In the present study, a frit inlet injection technique is applied to an asymmetrical flow FFF channel to allow a stopless sample injection method. With the application of a hydrodynamic relaxation technique using a frit inlet in asymmetrical flow FFF, the focusing process can be completely avoided, and the whole system operation can be done conveniently by one-step injection of sample materials to the sample inlet streamline directly, without incurring a serious band broadening. The schematic view of a frit inlet asymmetrical flow FFF (FIA-FlFFF) channel is shown in Figure 1a. When the frit stream encounters a sample stream of relatively low speed, an inlet splitting plane will be formed as shown in Figure 1b. The two streams will merge smoothly, and the frit stream will gradually displace the sample stream at the accumulation wall. During this process, sample materials are expected to be located below the inlet splitting plane by the compressing action of a high-speed frit flow, as illustrated in Figure 1b. Thus, sample relaxation can eventually be achieved by hydrodynamic means. If all particles are assumed to achieve complete hydrodynamic relaxation within the "relaxation segment" defined in Figure 1b, they will enter the separation segment smoothly, and their migration behavior will be similar to that expected for a typical asymmetrical channel system. The only difference between hydrodynamic relaxation and focusing relaxation in an asymmetrical system is the degree of broadening of the initial sample band. Comparison of the relaxation efficiency between hydrodynamic relaxation and focusing relaxation processes has not been provided in this work, but this will be further studied.

Compared to the limited use of a lowest axial flow rate in a frit inlet symmetrical flow FFF system, the present arrangement is more flexible in allowing the selection of a low axial flow rate condition which is suitable for low-retaining materials without the need of using a very high crossflow rate and for the reduction of injection amount due to the concentration effect. The use of a hydrodynamic relaxation process in asymmetrical flow FFF must first address the minimization of the initial band broadening. In the present work, the potential of the FIA-FlFFF system is discussed by examining the effect of the ratio of injection flow rate to frit flow rate on hydrodynamic relaxation with a number of polystyrene latex standards in both normal and steric/hyperlayer modes of FFF. To evaluate the relaxation efficiency, we need to consider some important factors which influence the formation of a starting band during the hydrodynamic relaxation.

The sample particles under hydrodynamic relaxation in symmetrical flow FFF will occupy a starting band with a length of h_s at or below the initial band height x_s as[13]

$$h_s = h_0\left[3\left(\frac{x_s}{w}\right)^2 - 2\left(\frac{x_s}{w}\right)^3\right] \quad (1)$$

where w is the thickness across the channel and h_0 is the travel distance of the sample band carried downstream in the channel by carrier liquid. (In a typical FFF channel, h_s is equal to h_0 since the initial band height x_s is always equal to w). The distance h_0 is given by $w\langle v\rangle/U$, where $\langle v\rangle$ is the average flow velocity along the axial direction and U is the average drift velocity across the channel (average velocity of crossflow in flow FFF).[11,13] When the initial band height x_s of particles is located close to the accumulation wall ($x_s/w \ll 1$) by an efficient operation of hydrodynamic relaxation, eq 1 approaches

$$h_s \cong h_0 3\left(\frac{x_x}{w}\right) \quad (2)$$

In the case of hydrodynamic relaxation with $\dot{V}_f \gg \dot{V}_s$, where $\dot{V}_s$ and $\dot{V}_f$ are the flow rates of sample substream and of the frit flow, respectively, x_s/w is approximately $(\dot{V}_s/3\dot{V}_f)^{1/2}$ (see ref 12). By using the relationship of $\langle v\rangle = \dot{V}/bw$ and $U = \dot{V}_c/bL$, eq 2 rearranges to

$$h_s = \frac{\dot{V}_s}{\dot{V}_f}\frac{\dot{V}}{\dot{V}_c}L \quad (3)$$

where $\dot{V}$ is the effective channel flow rate, $\dot{V}_c$ is the crossflow rate, b is the breadth of the channel, and L is the channel length. We expect a similar dependence of h_s on flow rates in the case of FIA-FlFFF, except that $\dot{V}$ should be replaced by $\dot{V}_{in}$ (equal to $\dot{V}_s + \dot{V}_f$) because relaxation takes place close to the inlet. Equation 3 suggests that a small ratio of sample flow rate to frit flow rate with a combined high crossflow rate is preferable in reducing h_s, which in turn minimizes relaxational band broadening in a FIA-FlFFF system.

EXPERIMENTAL SECTION

The frit inlet asymmetrical flow FFF channel was constructed in-house by replacing the top block of a conventional symmetrical flow FFF channel with a new lucite block having an inlet frit element carved into the top block near the channel inlet end. The original flow FFF channel was a kind gift from the FFF Research Center at the University of Utah (Salt Lake City, UT). A schematic drawing of the modified channel is shown in Figure 2. The length of the frit inlet wall is 3.1 cm from the channel inlet end, and the tip-to-tip channel length is 27.2 cm. A rectangular channel is cut

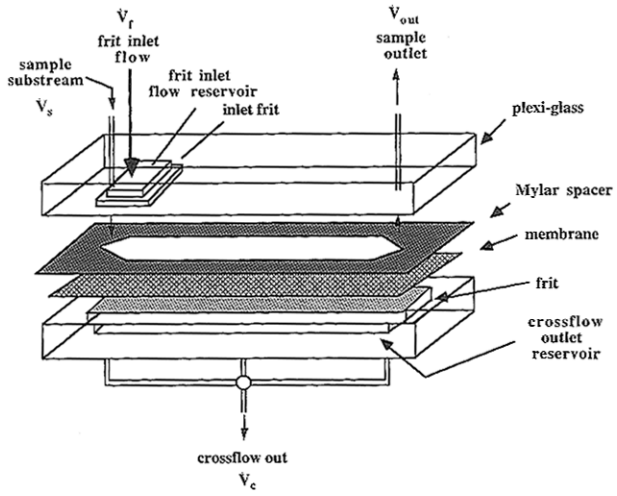

Figure 2. Schematic illustration of channel assembly of the frit inlet asymmetrical flow FFF system.

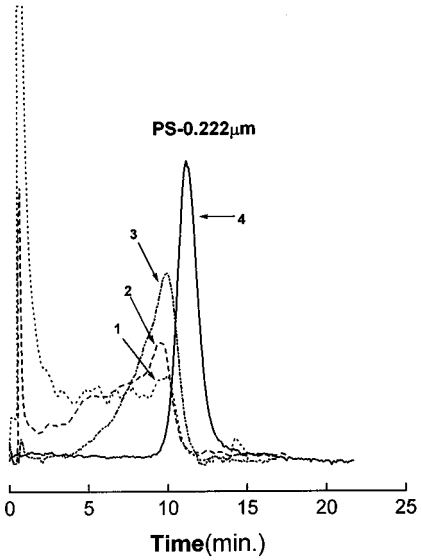

Figure 3. Effect of different levels of hydrodynamic relaxation on elution profiles of polystyrene 0.222 μm latex beads obtained at stopless flow injection in a frit inlet asymmetrical flow FFF system. The $\dot{V}_s/\dot{V}_f$ ratios of each profile correspond to (1) 100/0, (2) 50/50, (3) 30/70, and (4) 6/95, with the total inlet flow rate ($\dot{V}_s + \dot{V}_f$) of 1.64 mL/min for all cases. All runs are made with the same crossflow rate of $\dot{V}_c$= 1.09 and the same outflow rate $\dot{V}_{out}$ = 0.55 mL/min.

out of a 115 μm thick Mylar spacer with a fixed breadth of 2.0 cm, except for the triangular end at both the channel inlet and outlet. The geometrical volume of the channel space is 0.58 mL.

The membrane used as accumulation wall is of type YM-30, a regenerated cellulose having a MW cutoff of 30 000 (Amicon Co., Beverely, MA). Sample particles used for performance tests are polystyrene (PS) latex standards having mean diameters of 0.050, 0.135, 0.222, 0.300, 0.426, 3.004, 4.000, 4.991, 7.00, and 9.975 μm (Duke Scientific Co., Palo Alto, CA). The sample is introduced into the channel through the sample inlet hole by means of a 7725i Rheodyne sample injection valve (Rheodyne, Cotati, CA), equipped with a 20 μL sample loop. Injection amount is about <1 μg for each submicrometer-sized standard and about 2−4 μg for each supramicrometer-sized standard.

The carrier liquid for the separation of PS standards is purified (by reverse osmosis) deionized water containing 0.05% SDS to enhance sample dispersion and 0.02% sodium azide as a bactericide. It is filtered with a 0.45 μm membrane filter before use. Carrier solution is delivered to both the channel inlet and the frit inlet by using two different pumps: a Model 350 soft start pump (Bio-Rad Laboratories Inc., Hercules, CA) and a Vintage 2000 LC pump (OromTech, Seoul, Korea). Eluted particles are monitored with an M720 UV detector (Young-In Scientific Co. Ltd., Seoul, Korea) at 254 nm. The detector signal is transferred to a personal computer and saved with Chromastar II, a data acquisition and pump control software from OromTech.

RESULTS AND DISCUSSION

The efficiency in hydrodynamic relaxation in asymmetrical flow FFF is dependent on the initial band length, which is proportional to the ratio of sample substream flow rate, $\dot{V}_s$, to frit inlet flow rate, $\dot{V}_f$, according to eq 3. To drive sample components below the splitting plane by hydrodynamic forces, the frit flow rate must be high enough to compress them toward the accumulation wall. Figure 3 demonstrates the effect of $\dot{V}_s/\dot{V}_f$ on the elution profiles of 0.222 μm polystyrene latex beads in a frit inlet asymmetrical flow FFF channel. All runs are made at the same crossflow rate ($\dot{V}_c$ = 1.09 mL/min) and at the same channel outflow rate ($\dot{V}_{out}$ = 0.55 mL/min) by altering the ratio $\dot{V}_s/\dot{V}_f$ as (1) 100/0, (2) 50/50, (3) 30/70, and (4) 6/94. Since there is no incoming crossflow in an asymmetrical system, the total inlet flow rate, which is $\dot{V}_s + \dot{V}_f$ = 1.64 mL/min in Figure 3, is identical to the total outgoing flow rate, $\dot{V}_c + \dot{V}_{out}$. When there exists no frit inlet flow in profile 1, elution of 0.222 μm particles appears to be very broad, since particles do not experience hydrodynamic relaxation. This is a typical phenomenon which can be observed at stopless injection in an FFF channel without any proper relaxation process. When the ratio is decreased to 30/70 (0.49/1.15 mL/min as real flow rates), a single isolated peak appears but is still broad and distorted. The fronting in profile 3 means that particles are not completely relaxed to form a discrete equilibrium layer hydrodynamically due to the weak compression of frit inlet flow. Such broadness and asymmetry of the elution profile are removed when the ratio further decreases to 6/94 (0.10/1.54 mL/min) in profile 4. From the variation in degrees of hydrodynamic relaxation, it is clearly shown that the relaxational band broadening decreases gradually as the ratio decreases and that a relatively higher rate of frit inlet flow is necessary to achieve a complete relaxation in the present FIA-FlFFF system.

Similar results are obtained for the separation of a mixture of PS standards in Figure 4. The flow rates of crossflow and channel outflow are the same as those used in Figure 3. As expected from the previous run, separation of five different sizes of PS particles under $\dot{V}_s/\dot{V}_f$ = 50/50 results in a poor resolution in fractogram a of Figure 4. When the ratio is decreased to 6/94 in fractogram b, it can be seen that the individual particles are clearly separated with an excellent resolution. This result shows that hydrodynamic relaxation techniques are applicable to asymmetrical flow FFF in the separation of polydisperse particulate materials with the complete removal of the focusing process, which is otherwise a necessary step prior to the beginning of separation in asymmetrical flow FFF. In addition, a nearly baseline separation of five components is achieved at a 115 μm thick channel. According to a typical separation obtained by the conventional symmetrical flow FFF reported in ref 2, the complete baseline separation of particles up to 0.300 μm in diameter could be achieved under a

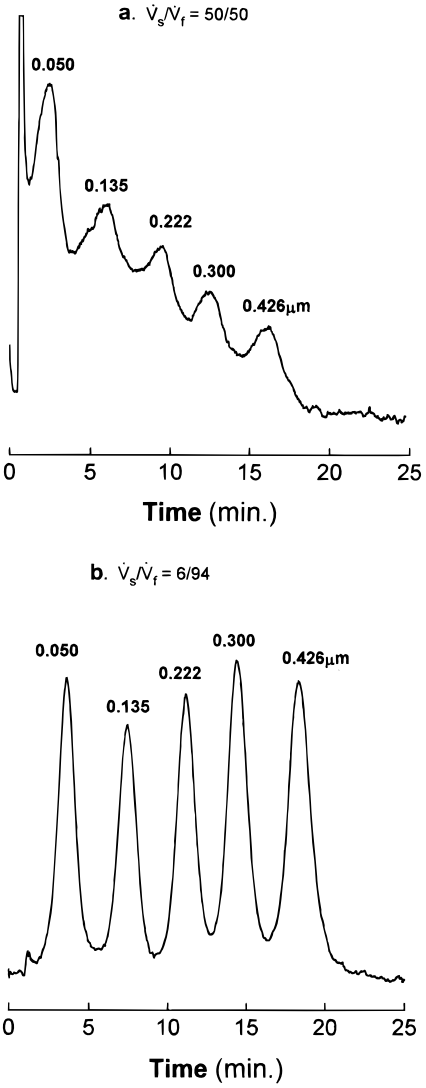

Figure 4. Separation of 0.050, 0.135, 0.222, 0.300, and 0.426 μm latex standards by stopless flow injection in a frit inlet asymmetrical flow FFF obtained at $\dot{V}_s/\dot{V}_f$ ratios of (a) 50/50 and (b) 4/96. Run conditions are the same as those used in Figure 2.

thicker channel ($w = 206\ \mu$m) within 30 min. A faster separation can be performed by using an intermediate channel thickness ($w = 154\ \mu$m);[3] however, an increased flow velocity in a thin channel causes broadening of emerging peaks with the increased steric effect. Since no previous work has been done to resolve submicrometer-sized particles by stopless flow injection in flow FFF, this is a promising result showing the performance of the frit inlet asymmetrical flow FFF channel in separating particulate materials.

Evaluation of the present system is continued with large steric particles ($\geq 1\ \mu$m) which are separated by the steric/hyperlayer operating mode of FFF. In this operation, particle diffusion has a negligible effect on retention because size factors predominantly govern the retention of large particles. Large particles protrude into the fast-moving streamlines of the parabolic flow profile in an FFF channel. Therefore, the elution order is the reverse of the normal mode of FFF: large particules elute earlier than the small ones. Figure 5 shows a separation of 10, 7, 5, 4, and 3 μm latex particles obtained at $\dot{V}_s = 0.15$, $\dot{V}_f = 7.20$, $\dot{V}_{out} = 0.91$, and $\dot{V}_c = 6.47$ mL/min using a frit inlet injection. In Figure 5, the ratio $\dot{V}_s/\dot{V}_f$ is significantly lowered to 2/98 in order to resolve the five

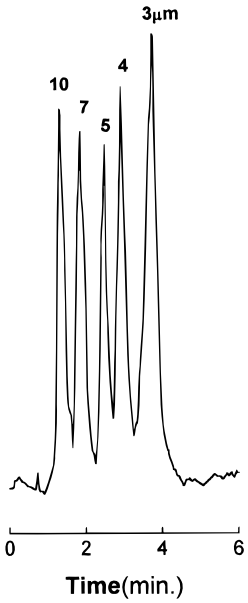

Figure 5. Steric/hyperlayer separation of 10, 7, 5, 4, and 3 μm latex particles by hydrodynamic relaxation in a frit inlet system with $\dot{V}_s/\dot{V}_f$ ratio of 2/98. Flow rates are $\dot{V}_s = 0.15$, $\dot{V}_f = 7.20$, $\dot{V}_c = 6.47$, and $\dot{V}_{out} = 0.91$ mL/min.

components successfully. Though the steric/hyperlayer separation in asymmetrical flow FFF using hydrodynamic relaxation is not fully resolved, it demonstrates a great capability of separating steric particles down to 3 μm at a high speed. Larger particles ($>10\ \mu$m) were not included in this run, since there was a difficulty in the passage of large particles through the back-pressure regulator, which has a needle valve. Upon looking at the fractogram, it appears that larger particles are resolved with the same run conditions, providing a good regulation of system back pressure. It is noted here that all sets of standard particles migrate with relatively narrow peaks throughout the run, while most steric/hyperlayer runs in flow FFF normally yield an increase in the peak width of late-emerging peaks.[13]

In this article, we have presented the first evaluation of hydrodynamic relaxation in frit inlet asymmetrical flow FFF as a potential separation technique. It is found that a relatively high speed of frit inlet flow is necessary to suppress the sample flow substream in order to achieve a successful hydrodynamic relaxation. The present work also shows the possibility of separating particles in asymmetrical flow FFF without using the focusing process. However, more work is needed to compare the two relaxation techniques (the hydrodynamic and the focusing relaxations) with the recovery study and to optimize the frit inlet asymmetrical flow FFF system for the separation of larger particles ($>10\ \mu$m). It is also desirable to further examine the band broadening phenomena characterizing the present system in the light of FFF theory. Using a shorter length inlet frit may induce a highly effective hydrodynamic relaxation, since reducing the area of the inlet frit element will result in a fast hydrodynamic relaxation of particles.

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GLOSSARY

b	channel breadth
h_0	bandwidth along channel due to normal relaxation
h_s	bandwidth after hydrodynamic relaxation
L	channel length
U	mean crossflow velocity (transverse velocity)
$\langle v \rangle$	mean axial flow velocity
$\dot{V}$	channel flow rate
$\dot{V}_c$	crossflow rate
$\dot{V}_f$	flow rate of carrier through frit inlet
$\dot{V}_{in}$	total inlet flow rate of carrier through sample inlet and frit inlet
$\dot{V}_{out}$	measured flow rate of carrier at channel outlet
$\dot{V}_s$	flow rate of sample inlet substream
w	channel thickness
x_s	elevation of splitting plane

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