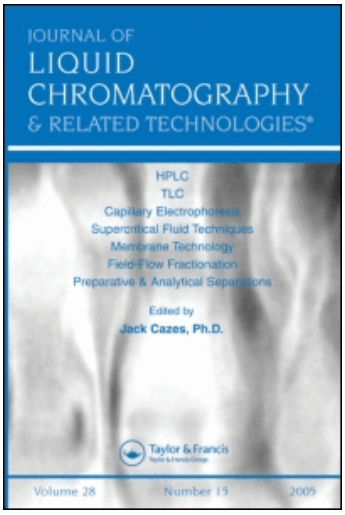

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Myeong Hee Moon[a]; Hansun Kwon[a]; Ilyong Park[a]
[a] Department of Chemistry, Kangnung National University Kangnung, Korea

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STOPLESS SEPARATION OF PROTEINS BY FRIT-INLET ASYMMETRICAL FLOW FIELD-FLOW FRACTIONATION

Myeong Hee Moon,* Hansun Kwon, Ilyong Park

Department of Chemistry
Kangnung National University
Kangnung, 210-702, Korea

ABSTRACT

The frit inlet asymmetrical flow field-flow fractionation is applied to the separation of proteins by using a stopless flow injection procedure. By utilizing a small permeable frit near the injection point in an asymmetrical flow FFF channel, sample materials injected to the flow streams can be hydrodynamically relaxed by the compressing action of high speed frit flow and the focusing/relaxation procedure can be bypassed. The separation efficiency of the frit inlet asymmetrical flow FFF channel is demonstrated with few protein standards by examining the influence of the ratio of injection flow rate to frit flow rate on the band broadening during relaxation.

INTRODUCTION

Flow field-flow fractionation (FlFFF) has shown its capability in the separation and characterization of particulate materials and macromolecules such as proteins, DNA, water soluble polymers, and etc.[1-7] As one of a wide

variety of FFF subtechniques, flow FFF utilizes an external force to have sample components retained in a thin rectangular channel. The force type used in flow FFF is the secondary flow (crossflow) of carrier liquid moving across the channel while the channel flow (separation flow) drives sample materials toward the end of the channel.[1-3]

When the cross flow is applied to a flow FFF channel, sample materials are driven toward the bottom of the channel wall. Due to the diffusive extrusion of sample materials away from the wall, particles or macromolecules under the external field are differentially accumulated at finite distances from the channel wall according to their diffusion rates which are closely related to their Stokes diameters. When the separation flow is applied to the sample components located at their equilibrium, they will be migrated at different velocities due to the parabolic nature of laminar flow between the thin channel walls and this leads to a separation of each other.

In practice, the whole system operation of a conventional symmetrical flow FFF is carried out by two consecutive steps; the sample relaxation process which is essential in most forms of FFF and is generally executed by applying crossflow only while the channel flow is stopped for a certain period of time, and the separation process which is begun by resuming the channel flow after the relaxation is completed.[8] However, in a conventional asymmetrical flow FFF system there is only one permeable wall on the bottom of the channel.[9] Since there is no influx of crossflow to the asymmetrical channel, sample relaxation is achieved by the focusing/relaxation process in which two counter directed flows (one from the channel inlet and the other from channel outlet) are focused at or below the sample loading point.[9]

By the focusing procedure, sample components can be accumulated into their equilibrium states as a narrow initial band. After the focusing/relaxation, separation process begins by applying the separation flow via the channel inlet. Since the incoming flow divides into cross flow and outflow, linear flow velocity gradually decreases at the end of the channel. This effect is expected to keep the sample band from spreading during migration and it possibly leads to a concentration effect of sample components entering the detector. The asymmetrical flow FFF channel has been utilized to separate proteins and their aggregates, plasmids, water soluble polymers, etc., at a high speed.[9-13] However, focusing/relaxation process is somewhat inconvenient in the system operation and the conversion of flows during the relaxation causes a baseline shift in the detector signal. Therefore, it is desired to simplify the system operation if possible.

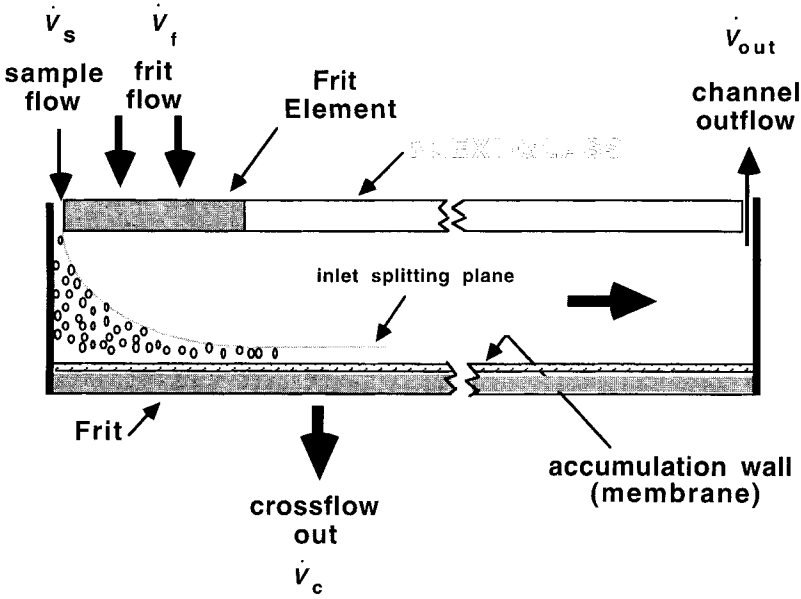

Figure 1. Schematic diagram of the side view of a frit inlet asymmetrical flow FFF (FIA-FlFFF) channel.

The present work utilizes a frit inlet relaxation technique to an asymmetrical flow FFF in order to simplify the relaxation procedure of asymmetrical flow FFF channel, and to facilitate the separation of macromolecules and particulate materials by stopless flow injection. The hydrodynamic relaxation technique has been applied to a conventional symmetrical flow FFF system earlier by using a frit inlet wall nearby the injection point.[7-8] In this work, a small frit element is implemented near the inlet of the plain wall of an asymmetrical channel. When sample materials are slowly introduced through the channel inlet of the so-called frit inlet asymmetrical flow FFF (FIA-FlFFF) channel, they are pushed toward the bottom of the channel wall by the incoming fast flow from the frit inlet wall. Thus, they can be hydrodynamically relaxed while they are continuously migrated down the channel. Relaxation pattern in FIA-FlFFF is expected to be similar to that observed in the frit inlet symmetrical system except that there is no crossflow into the channel. Figure 1 shows the side view of this channel, implemented with a small permeable frit wall at the inlet end of the upper channel wall. Once hydrodynamic relaxation is successfully achieved for the sample components entering the flow streams, they are expected to smoothly

migrate down the channel by being separated each other. With this channel design, a relaxation/focusing process can be bypassed and the system operation can be simplified by one-step injection without the stoppage of sample migration for relaxation. An initial evaluation of the channel was successful in the separation of polystyrene latex standards in both normal and steric/hyperlayer operating modes.[14] It is shown that the frit inlet asymmetrical channel is capable of separating latex particles without using the focusing process.

This paper, in memory of the late Professor J. Calvin Giddings, is written to demonstrate a possibility of separating proteins without the focusing/relaxation procedure, using a frit inlet asymmetrical flow FFF system. The importance of the ratio of injection flow to frit flow rate on the separation efficiency is discussed with the relaxational band broadening.

MATERIALS AND METHODS

The frit inlet asymmetrical flow FFF channel system is modified from a conventional flow FFF channel by substituting the top block with an in-house built lucite block which is implemented with a piece of frit for frit inlet. The channel has a tip-to-tip length of 27.2 cm and a frit inlet that is 3.1 cm long from the channel inlet. The channel space is made with a 254 μm thick Mylar sheet cut into a trapezoidal geometry having an initial breadth of 2.0 cm decreasing to 1.0 cm at the end of the channel. The inlet and outlet ends of the channel are treated as a triangle shape with 2.0 cm and 1.0 cm apart from the both ends, respectively.

Below the channel spacer, a membrane is used for the accumulation wall which enables the cross flow to pass through but keeps sample materials from penetration. The membrane material is YM-30, a regenerated cellulose, which has a molecular weight cutoff of 30,000 (Amicon Co., Beverly, MA, USA). Since the membrane is compressed by the channel spacer during the tightening of the channel blocks, the actual channel thickness is measured, by using the rapid breakthrough method, as 209 μm with the channel void volume of 1.05 mL.

The carrier solution used throughout the study was 0.1 M tris-HCl buffer solution (pH 7.8), prepared from water that is purified by reverse osmosis and deionized. For the delivery of the carrier solution to the channel inlet and frit inlet, two HPLC pumps are used: a Model 350 Soft Start Pump (Bio-Rad

Seoul, Korea). The eluted proteins were monitored by a model 720 UV detector (Young-In, Seoul, Korea) at a wavelength of 280 nm. The two oulet (channel and cross flow outlets) flow rates are controlled by a fine metering needle valve, Whitey SS-22RS2 (Crawford Fitting Co., Solon, OH, USA) located after the detector.

RESULTS AND DISCUSSION

Figure 2 shows the separation of three proteins: ovalbumin, γ-globulin, and thyroglobulin, by using frit inlet asymmetrical flow FFF under the different levels of hydrodynamic relaxation. The three runs are obtained at the same condition as outflow rate $\dot{V}_{out}$ of 0.41mL/min and crossflow rate ($\dot{V}_c$) of 5.78mL/min by controlling the ratio of sample flow rate ($\dot{V}_s$) to the frit flow rate ($\dot{V}_f$). When the ratio $\dot{V}_s / \dot{V}_f$ is set at 48/52 (2.91/3.10 mL/min in real flow rate) in run **a** of Figure 2, serious band broadening during the relaxation is observed for all proteins as broad and diffused peaks. This represents that hydrodynamic relaxation of proteins is not achieved at all with the current injection condition used in run **a**.

As $\dot{V}_s / \dot{V}_f$ is decreased to 8/92, it appears with individual peaks, but they are still broad. When the ratio is further decreased to 4/96 (0.24/5.90 mL/min), a better separation is obtained for the three protein samples with an almost baseline resolution. The small peak right after the peak of γ-globulin is presumed to be dimers of γ-globulin. Figure 2 demonstrates how the ratio of sample flow rate to frit flow rate is important in achieving a good relaxation by hydrodynamic means.

Figure 3 shows the separation of transferrin and apoferritin with the presumed aggregates obtained at the same run condition used in Figure 2 except that the ratio of sample flow rate to frit flow rate is slightly changed to 0.21/6.0 mL/min. Separation of these proteins is successfully achieved in frit inlet asymmetrical flow FFF with the clear identification of each component.

The small peaks right after each monomer peak are presumed to be from their dimers, and a flat peak shown at the very last in Figure 3 from trimers of apoferritin. The identification of the peak of presumed to be aggregates was not confirmed by other means. However, the relative increase in the retention time of the presumed dimer peak from that of the monomer is observed to be

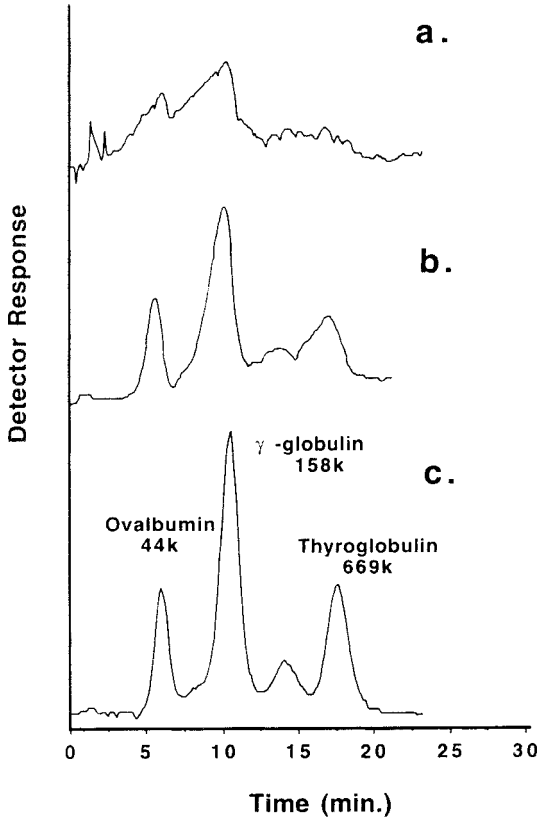

Figure 2. Elution profiles of proteins by varying the ratio of sample flow rate ($\dot{V}_s$) to frit flow rate ($\dot{V}_f$) by FIA-FlFFF: the ratios $\dot{V}_s$ / $\dot{V}_f$ are a) 48/52 (2.91/3.10 mL/min in real flow rates), b) 8/92 (0.80/5.40 mL/min.), and c) 4/96 (0.24/5.90mL/min.). Separation condition for all runs is fixed at $\dot{V}_{out}$ = 0.41 and $\dot{V}_c$ = 5.78 mL/min.

dimers, and a flat peak shown at the very last in Figure 3 from trimers of apoferritin. The identification of the peak of presumed to be aggregates was not confirmed by other means. However, the relative increase in the retention time of the presumed dimer peak from that of the monomer is observed to be about 1.35-1.40 which is very close to the reported value 1.40 reported earlier by a conventional asymmetrical flow FFF.[9] The presumed aggregates may be traced as follows.

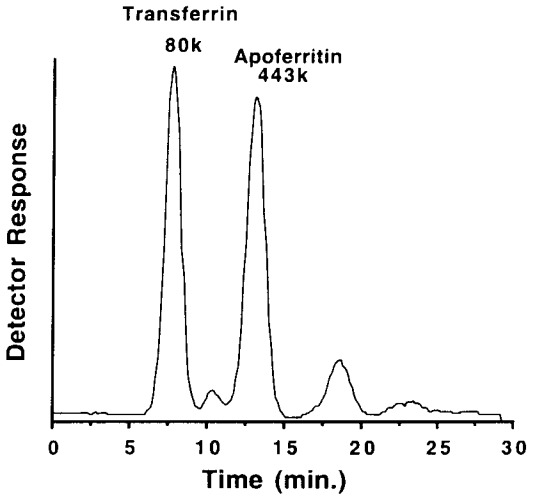

Figure 3. Separation of transferrin and apoferritin and their aggregates by FIA-FlFFF. Run condition is $\dot{V}_s / \dot{V}_f$ = 0.21/6.0 mL/min. and $\dot{V}_c / \dot{V}_{out}$ = 5.78/0.41 mL/min.

Figure 4 shows the correlation plot of log t_r vs. log M of protein monomers (marked as filled circles) shown in Figures 2 and 3, and the superimposed data points (marked as open triangles) of presumed aggregates. The correlation is done with the monomers only, and the straight line correlation is reasonably good with a slope of 0.37, which is slightly higher than the theoretical value of 0.33 in flow FFF,[7] but is somewhat lower than the molecular weight selectivity of 0.49 reported in a work done by conventional asymmetrical flow FFF.[9] The latter value was based on the calculation of exponent b in $D=AM^{-b}$ (D is diffusion coefficient of the protein, A is a constant, and M is the molecular weight of protein) from the ratio 1.40.[9] The retention time data of presumed aggregates appear to fit the correlation curve quite well. From these considerations, they could be assumed as the dimer peaks and a trimer peak. It is also demonstrated that the frit inlet asymmetrical flow FFF system behaves similarly to the conventional asymmetrical system in fractionating proteins and their aggregates.

In order to examine the influence of the flow rate ratio $\dot{V}_s / \dot{V}_f$ on the band broadening during the hydrodynamic relaxation in asymmetrical flow FFF, experimental plate heights are examined at various injection conditions. For a well retained peak provided with a complete relaxation, the relaxational

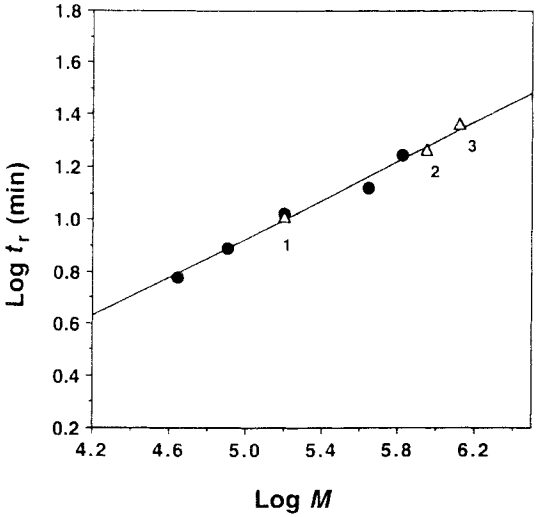

Figure 4. Plot showing correlation between retention time, t_r, and protein molecular weight, M for the monomers shown in Figure 2 and 3. The data points of presumed dimer and trimer peaks (marked as open triangles) are superimposed over the correlation plot. 1. Dimer of transferrin; 2 and 3 are the dimer and trimer of apoferritin, respectively.

contribution to total band broadening can be minimized. In case of using a hydrodynamic relaxation, incomplete relaxation in FIA-FlFFF leads to a substantial increase in the total band broadening of an eluted peak, as observed in Figure 2, which results in the increase of the observed plate height.

Figure 5 shows the plot of observed plate heights of apoferritin vs. the $\dot{V}_s / \dot{V}_f$ ratio. The data points marked as triangles show the plate height data obtained at $\dot{V}_{out}$ = 1.61 and $\dot{V}_c$ =7.45 mL/min, and the lower set (marked as open circles) obtained at $\dot{V}_{out}$ = 0.42 and $\dot{V}_c$ =5.81 mL/min. For the case of upper data set, observed plate height is minimized when the $\dot{V}_s / \dot{V}_f$ ratio decreases to about 3/97. It is shown that the band broadening gradually increases as the ratio increases. When the ratio decreases to a very low level (on the left side of the plot), an unexpected increase in band broadening is observed, due to the relatively slow introduction of sample materials to the sample inlet ($\dot{V}_s$ =0.10 and $\dot{V}_f$ = 8.96mL/min) compared to the fast frit flow.

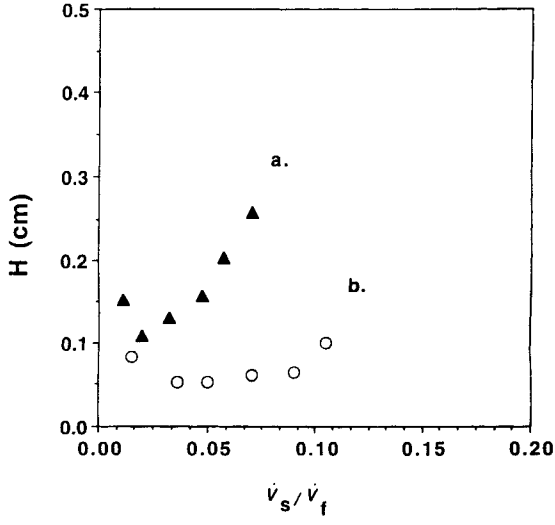

Figure 5. Plot of plate height, H, versus the $\dot{V}_s / \dot{V}_f$ ratio for apoferritin obtained at two different separation conditions; a) $\dot{V}_c / \dot{V}_{out}$ = 7.45/1.61 mL/min and b) $\dot{V}_c / \dot{V}_{out}$ = 5.81/0.42 mL/min.

Compared to the limited usage in optimum hydrodynamic relaxation at the upper set, a relatively strong field strength condition gives more flexibility in selecting the optimum ratio as illustrated in the lower data set. In this case, the outflow rate is decreased to 0.42 mL/min along with the reduction of crossflow rate to 5.81mL/min but $\dot{V}_c / \dot{V}_{out}$ ratio is increased to 13.8 from 4.63 for the upper run condition. In a conventional asymmetrical channel, an increase in the $\dot{V}_c / \dot{V}_{out}$ ratio leads to an improvement in separation efficiency. Thus, the increase in effective field strength in FIA-FlFFF appears to provide less chance of broadening of the initial sample band during hydrodynamic relaxation. The plate heights appear to be minimized in the region of $\dot{V}_s / \dot{V}_f$ from approxiamtely 4/96 up to 8/92 in extreme.

The maximum plate numbers obtained at $\dot{V}_s / \dot{V}_f$ = 4/96 is about 500. It is noted, in Figure 5, that the optimum $\dot{V}_s / \dot{V}_f$ to be used is not fixed and is dependent on experimental run conditions such as $\dot{V}_c / \dot{V}_{out}$ ratio.

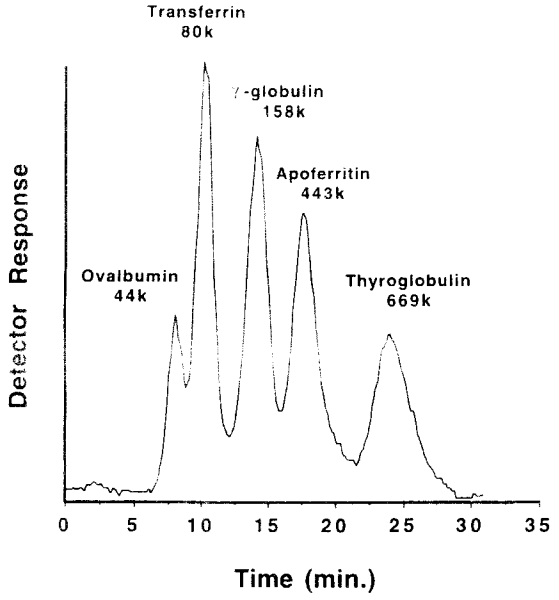

Figure 6. Separation of five proteins by AFI-FlFFF obtained at $\dot{V}_s = \dot{V}_{out} = 0.22$ mL/min. and $\dot{V}_f = \dot{V}_c = 5.80$ mL/min.

An extreme case of run condition is employed in Figure 6, as the outflow rate is adjusted to be the same as the sample flow rate so that all frit flow can be served as crossflow out. Figure 6 illustrates the separation of five proteins using FIA-FlFFF obtained at $\dot{V}_s = \dot{V}_{out} = 0.22$ and $\dot{V}_f = \dot{V}_c = 5.80$ mL/min. The resolution is reasonably acceptable to identify each protein but it takes about 30 minutes for the entire separation, which is somewhat slow. However, the enhancement of separation speed may be achieved if a thinner channel is properly used or the length of inlet frit is reduced. The latter modification leads to a reduction of area of frit inlet element and this may possibly increase the efficiency of hydrodynamic relaxation. In this report, it is demonstrated that FIA-FlFFF is capable of separating proteins and their aggregates by using a hydrodynamic relaxation technique. We note that a successful hydrodynamic relaxation requires a proper selection of $\dot{V}_s / \dot{V}_f$ which falls in the range of 3/97~6/94. This means that a relatively high frit flow rate must be used to efficiently suppress the sample stream toward the accumulation wall and to lead to their equilibrium hydrodynamically.

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