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## Effect of Inlet Frit Lengths on the Hydrodynamic Relaxation Efficiency in Frit Inlet Asymmetrical Flow Field-Flow Fractionation

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### ABSTRACT

The efficiency of a frit inlet asymmetrical flow field-flow fractionation (FI-AFIFFF) channel has been evaluated by varying the length of the inlet frit element. In a FI-AFIFFF channel, a high speed frit flow is introduced through the inlet frit to provide hydrodynamic relaxation of the incoming sample stream from the channel inlet. The experimental plate heights and peak recovery values are examined for three different FI-AFIFFF channels by varying field strengths and the channel membrane materials. It has been found that the length of the inlet frit element influences the performance of hydrodynamic relaxation, as well as peak recovery in the FI-AFIFFF channel system. Experimental plate height data show that the hydrodynamic relaxation itself occurs more efficiently when the length of inlet frit is longer. While an FI-AFIFFF channel having a longer

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inlet frit (4.2 cm long) gives a good relaxation, it shows less efficiency in considering peak recovery over a range of the field strengths examined in this study. A similar tendency is observed when channel membrane is varied. By considering the efficiency of hydrodynamic relaxation and sample recovery during elution, an FI-AFIFFF channel of an intermediate length of inlet frit (3.2 cm) is shown, from experiments, to be optimum.

*Key Words:* Frit inlet asymmetrical flow FFF; Field-flow fractionation; Particle separation; Effect of inlet frit length; Hydrodynamic relaxation.

## INTRODUCTION

Frit inlet asymmetrical flow field-flow fractionation (FI-AFIFFF)<sup>[1,2]</sup> is a modified form of the flow field-flow fractionation (flow FFF or FIFFF) technique that is known as an analytical separation technique for macromolecular species and nano or micron sized particulate materials.<sup>[3-5]</sup> Since a small inlet frit element is installed at the beginning end of the depletion wall of an asymmetrical flow field-flow fractionation (AFIFFF) channel in which there is no crossflow influx toward the channel, sample introduction is made through the channel inlet normally at a very low flow rate, while a high speed frit flow enters the channel through the inlet frit element.<sup>[1,2]</sup> The diagram of a FI-AFIFFF channel structure and a side view of the channel inlet region are shown in Fig. 1. The major role of frit flow entering at a high speed relative to the sample stream rate, is to compress incoming sample components toward the accumulation wall of the channel, through which part of the total flow (frit flow plus sample flow) exits across the channel, through a semi-permeable membrane layered above a porous frit wall [see Fig. 1(b)]. The rest of the flow drives sample components down the channel toward the detector.

When sample components are pushed toward the accumulation wall, they will experience diffusive forces against the wall according to their hydrodynamic sizes. Therefore, a sample component driven toward the accumulation wall will find an equilibrium slightly away from the wall. At the equilibrium position, the two counteracting forces balance each other and the sample relaxation can be achieved hydrodynamically while they are continuously migrating down the channel for separation. The main advantage of using a frit inlet relaxation is to bypass stopflow relaxation,<sup>[6]</sup> or focusing/relaxation procedures,<sup>[7]</sup> that are normally carried out by stopping migration flow for a certain period of time to establish equilibrium conditions of sample components. These are the prerequisites of conventional flow FFF channel systems in order to avoid a serious broadening of the migrating sample band when it is processed without the relaxation procedure. By using a



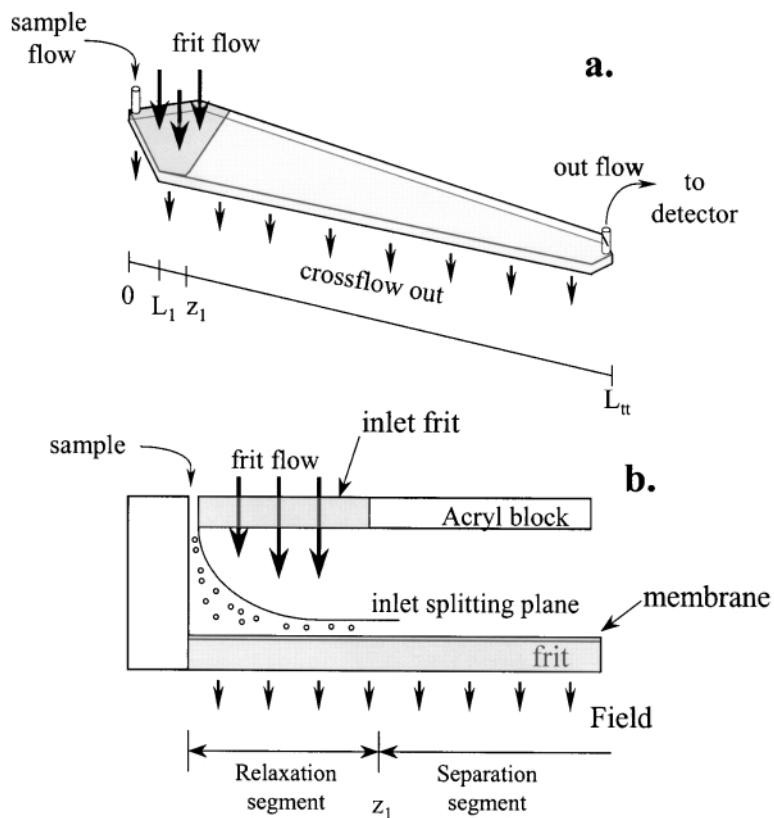


Figure 1. (a) Schematic diagram of frit inlet asymmetrical flow FFF channel and (b) the side view of channel inlet region.

hydrodynamic relaxation in FI-AFIFFF, sample injection, relaxation, and separation processes can be smoothly operated with a minimized risk of sample adhesion at the channel membrane.<sup>[8,9]</sup>

However, band broadening always occurs during hydrodynamic relaxation when a FI-AFIFFF system is used, since it adopts the stopless flow injection method. A somewhat smaller band broadening can be normally obtained by the focusing/relaxation method in a conventional AFIFFF channel.<sup>[10]</sup> Minimization of band broadening during hydrodynamic relaxation is likely to be one of the most important tasks for a successful separation in FI-AFIFFF. Previous studies have shown that an efficient hydrodynamic relaxation can be somehow obtained by maintaining a ratio of sample stream rate to frit flow rate less than or equal to 0.05.<sup>[1,11]</sup>





Recently, it has been demonstrated that experimental plate height values obtained by hydrodynamic relaxation can be minimized when the ratio of outflow rate to the total flow rate (outflow rate plus crossflow rate) is kept around 0.1.<sup>[10]</sup> While the focusing relaxation process in an asymmetrical channel system provides higher relaxation efficiency with flexibility in selecting high speed separation conditions than hydrodynamic relaxation, FI-AFIFFF has been shown to perform well under higher field strength conditions, with higher sample recoveries, than a conventional AFIFFF channel.<sup>[10]</sup> Moreover, it has been shown that the lower separation speed can somehow be overcome by introducing a straightforward and easily implemented field program to FI-AFIFFF, by circulating crossflow to frit flow with a simultaneous decrease in flow rate.<sup>[12,13]</sup>

Since hydrodynamic relaxation is influenced by the strength and duration of the compressive forces of frit flow, it is interesting to investigate the relaxation efficiency by varying the length of the inlet frit element at the accumulation wall. In this work, experimental plate height and their peak recovery values are examined at three FI-AFIFFF channels having different inlet frit lengths. The influence of the channel membranes on the peak recovery values is also investigated using a few protein standards at different field strengths.

## EXPERIMENTAL

Three different FI-AFIFFF channels are utilized in this study. Three channels can be constructed, one at a time, by assembling the same accumulation wall blocks with three different depletion wall blocks. These blocks were built in house by using small pieces of porous ceramic frit with varying lengths. Construction of the channel blocks was accomplished using the same method described in earlier studies.<sup>[10-13]</sup> The lengths of inlet frit measured from the channel inlet end to the end of the relaxation segment at the depletion wall [see Fig. 1(b)],  $z_1$ , are 2.2, 3.2, and 4.2 cm. The channel space was made by cutting a 250  $\mu\text{m}$  thick Mylar spacer in a ribbonlike shape. The tip-to-tip channel length is 28.0 cm, the initial channel breadth is 2.0 cm, which is trapezoidally decreased to a final breadth of 1.0 cm. At the accumulation wall, a membrane was placed above the frit to keep sample materials from penetrating through the wall.

In this work, three different membranes were used and they were basically the same material, but differ slightly from each other in terms of molecular weight cut off (MWCO) values and manufacturers: YM-10 (MWCO: 10 kDa) from Amicon Co. (Beverly, MA), PLCGC (MWCO: 10 kDa), and PLCCC (MWCO: 5 kDa) from Millipore Corp. (Danvers, MA). The last two membranes have different self sealing backing materials to prevent leaking.





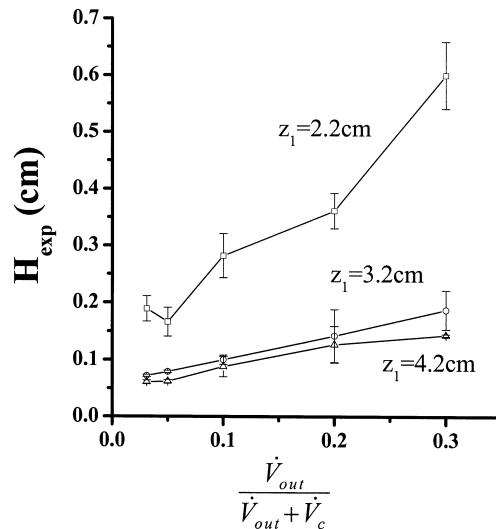
Carrier solution for the flow FFF separation is 0.1 M Tris-HCl buffer adjusted at pH = 7.4. The solution is prepared from ultrapure water (>18 M $\Omega$ ) and is filtered through a membrane filter with a pore size of 0.45  $\mu$ m prior to use. The carrier solution is delivered to both channel inlet (sample flow) and frit inlet (frit flow) through two independent HPLC pumps: model LC10 from Shimadzu (Tokyo, Japan). The outflow from the channel outlet was lead to the detector, a model 720 UV detector from Young-Lin Scientific Co. (Seoul, Korea), and the eluting protein samples were monitored at a wavelength of 280 nm. The cross flow rate was controlled by pressurizing the outflow with a needle valve at the end of the detector. Protein standards, used in this study, were obtained from Sigma (St. Louis, MO): cytochrome-C (12.4 kDa), BSA (66 kDa), apoferritin (443 kDa), and thyroglobulin (667 kDa). Sample injection was made with a model 9125 loop injector from Rheodyne (Cotati, CA), having a 10  $\mu$ L sample loop.

## RESULTS AND DISCUSSION

The influence of inlet frit length in a FI-AFIFFF channel on the broadening of an eluted sample peak has been investigated by measuring the experimental plate height data of albumin (BSA, 66 kDa). Figure 2 shows the  $H_{\text{exp}}$  obtained by increasing the ratio of outflow rate to the total out flux rate (sum of outflow rate and crossflow rate),  $\dot{V}_{\text{out}}/(\dot{V}_{\text{out}} + \dot{V}_{\text{c}})$ , at three different FI-AFIFFF channels. All runs are made at the same sample flow rate,  $\dot{V}_{\text{s}} = 0.20 \text{ mL min}^{-1}$ . The experimental plate height values plotted in Fig. 2 show that a FI-AFIFFF channel with a shorter inlet frit ( $z_1 = 2.2 \text{ cm}$ ) gives a broader sample band at the elution than the channels with longer inlet frits. It can be thought that a more complete hydrodynamic relaxation could be achieved with a shorter inlet frit, since sample components can be quickly compressed toward the accumulation wall by the relatively faster flow through the smaller frit. However, the results show that the longer inlet frit provides better efficiency in hydrodynamic relaxation, opposite to the simple expectation. This can be explained, by noting that while sample components entering the channel with a shorter inlet frit are being compressed toward the accumulation wall, they will be forced toward the end of the channel more quickly than if they are in a channel with a longer inlet frit. The local migration flow rate within the triangular region of the relaxation segment of an FI-AFIFFF channel [see Fig. 1(a)],  $\dot{V}_l(z)$ , is expressed as the sum of the sample flow rate  $\dot{V}_{\text{s}}$  and the fraction of frit flow rate  $\dot{V}_{\text{f}}$  up to any arbitrary point  $z$  in the segment  $l$ , which denotes

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**Figure 2.** Plot of experimental plate height of BSA vs. the ratio of outflow rate to the total flow rate (sum of outflow and crossflow rates) obtained with three different FI-AFIFFF channels.

the segment under the triangular segment, minus the fraction of crossflow rate  $\dot{V}_c$  exiting the channel up to this point:<sup>[2]</sup>

$$\dot{V}_l(z) = \dot{V}_s + \frac{\dot{V}_f A_f(z)}{A_f} - \frac{\dot{V}_c A_l(z)}{A_c} \quad (0 \leq z \leq L_1) \quad (1)$$

where  $L_1$  is the distance between the channel inlet and the end of the triangular piece,  $A_f$  and  $A_c$  are the area of inlet frit element and the total area of channel accumulation wall, respectively. From Eq. (1), it is found that when a shorter inlet frit is used, the area of an inlet frit element,  $A_f$ , becomes smaller and this leads to an increase in the local migration flow rate at any point  $z$  along the channel axis. Thus, the initial sample band during the hydrodynamic relaxation process becomes broader when the inlet frit element is shortened. Figure 2 also suggests that it would be better to keep the ratio of outflow rate to the total flow rate smaller than 0.1 for an efficient separation. The results shown in Fig. 2 were obtained by using a PLCCC membrane (MWCO = 5 kDa). In order to examine the influence of the membrane type on efficiency, two other membranes composed of materials similar to the regenerated cellulose but different in MWCO or manufacturer, are tested by using a FI-AFIFFF channel having an inlet frit of 3.2 cm long. The experimental plate height data are listed in Table 1

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**Table 1.** Experimental plate height ( $H$ ) values for apoferritin obtained at three different membrane materials using 3.2 cm long inlet frit in FI-AFIFFF.

$\dot{V}_{\text{out}}$ (mL min <sup>-1</sup> )	$\dot{V}_{\text{out}}/(\dot{V}_{\text{out}} + \dot{V}_{\text{c}})$	Height (cm)		
		PLCGC	PLCCC	YM-10
0.13	0.03	0.070 ± 0.003	0.072 ± 0.002	0.060 ± 0.002
0.21	0.05	0.077 ± 0.005	0.079 ± 0.002	0.073 ± 0.006
0.42	0.10	0.104 ± 0.009	0.100 ± 0.001	0.090 ± 0.013
0.84	0.20	0.141 ± 0.019	0.142 ± 0.046	0.145 ± 0.037
1.26	0.30	0.181 ± 0.017	0.187 ± 0.034	0.176 ± 0.030

*Note:* All runs are made at a fixed  $\dot{V}_{\text{s}} = 0.20$  mL min<sup>-1</sup> with an injection amount of 4.0 µg.

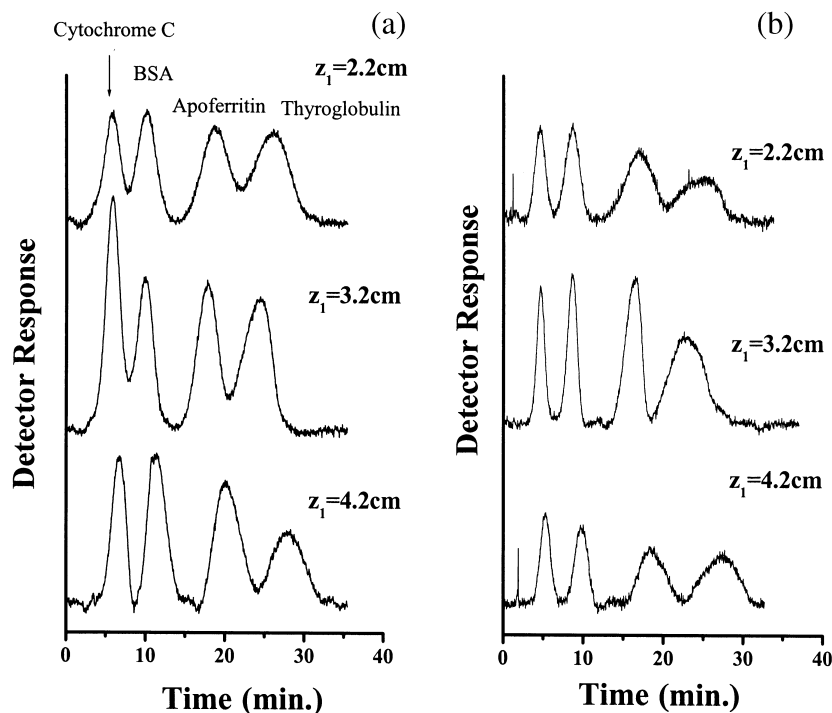
and they were obtained the same way as shown in Fig. 2. As shown in Table 1, plate height values obtained at each flow rate condition for PLCGC (MWCO = 10 kDa) and PLCCC membranes are similar to each other, while they differ only in MWCO. Data obtained by using YM-10 seems to be slightly smaller than those obtained with the other two, but the difference is not significant (within about 5 ~ 10%).

Separation resolution of protein mixtures has been examined at three channel systems under various field strength conditions. Figure 3 shows the comparison of fractograms obtained at each FI-AFIFFF channel, only under two different flow rate conditions: (a)  $\dot{V}_{\text{s}}/\dot{V}_{\text{f}} = \dot{V}_{\text{out}}/\dot{V}_{\text{c}} = 0.10/2.0$  (mL min<sup>-1</sup>) and (b)  $\dot{V}_{\text{s}}/\dot{V}_{\text{f}} = \dot{V}_{\text{out}}/\dot{V}_{\text{c}} = 0.20/4.0$  (mL min<sup>-1</sup>). As the fractograms in Fig. 3(a) were obtained at a lower frit flow rate, the separation of four protein mixtures in a channel using a shorter inlet frit ( $z_1 = 2.2$  cm) appears to have been barely achieved. The resolution appears to increase as the inlet frit length increases, as shown in Fig. 3(a). It is also shown, that the retention times of each component in the channel ( $z_1 = 4.2$  cm) have increased slightly due to the decrease of the local migration flow rate at the relaxation segment, as found in Eq. (1). When the frit flow rate is increased by twice in Fig. 3(b), as well as crossflow rate, it is clearly shown that the separation times in each channel are substantially reduced from those achieved under a low frit flow rate condition, due to the decrease in the effective migration flow rate,  $\dot{V}_{\text{eff}}$ , which is also dependent on the crossflow rate. Under a lower field strength condition in Fig. 3(a), the channel using a longer frit seems to give a better resolution when separating four protein components. However, when the frit flow rate and field strength are increased simultaneously, the channel having a 3.2 cm long frit inlet provides a better separation with sharper peaks compared to the other channels. This means that under a slow frit flow rate, a longer inlet frit provides a better

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**Figure 3.** Comparison of separation fractograms obtained at each FI-AFIFFF channel under different flow rate conditions; (a)  $\dot{V}_s/\dot{V}_f = \dot{V}_{out}/\dot{V}_c = 0.10/2.0$  ( $\text{mL min}^{-1}$ ) and (b)  $\dot{V}_s/\dot{V}_f = \dot{V}_{out}/\dot{V}_c = 0.20/4.0$  ( $\text{mL min}^{-1}$ ).

hydrodynamic relaxation of sample components leading to a decrease in band broadening. When frit flow rate is increased, separation of each component is shown to be achieved more completely. However, a smaller inlet frit ( $z_1 = 2.2$  cm) appears to give a peak broadening for thyroglobulin in Fig. 3(b), while the separation of the first two peaks are well resolved. This is because the initial sample band is spread more by the increased axial flow velocity exerted on the sample components undergoing hydrodynamic relaxation. The spreading effect becomes larger for large MW components, which are expected to travel closer to the accumulation wall for their equilibrium.

By comparing the fractograms obtained at the flow rate regions used in Fig. 3, it is thought that the intermediate channel ( $z_1 = 3.2$  cm) appears to provide a better separation among the three channels. In order to check the peak recovery, peak area of BSA is measured by varying field strength conditions and membrane materials at three different channels and these are



listed in Table 2. For all run conditions, cross flow rate is adjusted to be equal to the frit flow rate. As the field strength increases, peak recovery values appear to decrease throughout the experiments. However, the peak recovery values at YM-10 membrane in Table 2(a) are shown to be larger at the intermediate channel ( $z_1 = 3.2$  cm), which is similar to the results for the other two membranes in Table 2(b) and (c). Another noteworthy difference in the peak recovery values occurs among membrane types. YM-10 and PLCGC are basically the same regenerated cellulose with MWCO of 10 kDa and show a similar performance in peak recovery though they are from different sources. However, PLCCC, which has MWCO of 5 kDa seems to provide the lowest recovery values among the three membranes. This might come from the nonuniformity in the flow penetration through pores of membrane.

In this study, the effect of inlet frit length on hydrodynamic relaxation has been examined by studying experimental plate height and peak recovery data. It is found, that the length of the inlet frit in FI-AFIFFFs channel is one of the

**Table 2.** Effect of length of inlet frit on peak recovery measured with BSA.

$\dot{V}_s/\dot{V}_{out} = \dot{V}_f/\dot{V}_c$ (mL min <sup>-1</sup> )	Recovery (%)		
	$z_1 = 2.2$ cm	$z_1 = 3.2$ cm	$z_1 = 4.2$ cm
(a) YM-10 (MWCO: 10 kDa)			
1.0	73.2 ± 2.2	84.2 ± 2.7	75.8 ± 2.3
2.0	71.6 ± 4.5	81.8 ± 2.0	74.3 ± 3.6
3.0	66.6 ± 3.0	81.1 ± 2.9	73.2 ± 1.4
4.0	69.6 ± 3.4	77.3 ± 2.3	70.7 ± 2.1
5.0	67.2 ± 3.9	74.9 ± 4.3	68.5 ± 2.9
(b) PLCGC (MWCO: 10 kDa)			
1.0	69.2 ± 2.7	87.2 ± 3.1	75.6 ± 1.3
2.0	68.0 ± 0.5	87.3 ± 1.5	75.5 ± 2.4
3.0	66.8 ± 3.2	86.2 ± 2.5	75.2 ± 2.2
4.0	64.3 ± 1.3	86.2 ± 0.8	73.6 ± 4.4
5.0	64.1 ± 2.8	85.5 ± 1.0	69.7 ± 4.3
(c) PLCCC (MWCO: 5 kDa)			
1.0	65.9 ± 3.8	68.3 ± 3.6	65.9 ± 2.0
2.0	61.8 ± 3.1	63.1 ± 3.4	62.6 ± 0.6
3.0	57.5 ± 4.3	62.5 ± 3.0	62.2 ± 2.3
4.0	56.3 ± 4.3	62.3 ± 2.4	59.8 ± 4.1
5.0	56.7 ± 4.7	61.1 ± 2.6	58.7 ± 3.9

*Note:* Three different membrane materials are compared. All experiments are made at  $\dot{V}_s/\dot{V}_{out} = \dot{V}_f/\dot{V}_c$ .





contributing factors in controlling band broadening during hydrodynamic relaxation and peak recoveries. While band broadening during hydrodynamic relaxation can be minimized with a longer inlet frit, sample recovery is higher with the channel having an inlet frit of intermediate length. Since sample relaxation in a FI-AFIFFF channel is dependent on the hydrodynamic nature of two different flow streams entering the channel, the effect of the inlet frit length may significantly influence the initial stage of sample relaxation. Separation of highly retaining components will be more seriously affected by the successful hydrodynamic relaxation within the relaxation segment of the frit inlet channel. For an improved sample recovery along with increased speed, it is desirable to adopt a field programming technique using a longer inlet frit.

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