Ilyong Park[1]
Ki-Jung Paeng[1]
Dukjin Kang[2]
Myeong Hee Moon[2]

[1]Department of Chemistry, Yonsei University, Wonju, Kangwon-do, Korea
[2]Department of Chemistry, Yonsei University, Seoul, Korea

Performance of hollow-fiber flow field-flow fractionation in protein separation

Since hollow-fiber flow field-flow fractionation (HF FlFFF) utilizes a cylindrical channel made of a hollow-fiber membrane, which is inexpensive and simple in channel assembly and thus disposable, interests are increasing as a potential separation device in cells, proteins, and macromolecules. In this study, performance of HF FlFFF of proteins is described by examining the influence of flow rate conditions and length of fiber (polyacrylonitrile or PAN in this work) on sample recovery as well as experimental plate heights. The interfiber reproducibility in terms of separation time and recovery was also studied. Experiments showed that sample recovery was consistent regardless of the length of fiber when the effective field strength (equivalent to the mean flow velocity at the fiber wall) and the channel void time were adjusted to be equivalent for channels of various fiber lengths. This supported that the majority of sample loss in HF FlFFF separation of apoferritin and their aggregates may occur before the migration process. It is finally demonstrated that HF FlFFF can be applied for characterizing the reduction in Stokes' size of low density lipoproteins from blood plasma samples obtained from patients having coronary artery disease and from healthy donors.

Key Words: Fiber length effect; Hollow-fiber flow field-flow fractionation; Interfiber reproducibility; Lipoprotein separation; Low density lipoprotein

Received: March 21, 2005; revised: May 9, 2005; accepted: May 19, 2005

DOI 10.1002/jssc.200500125

1 Introduction

Hollow-fiber flow field-flow fractionation (HF FlFFF) is a variant of flow FFF, an analytical separation method that is capable of fractionating and characterizing particles, cells, proteins, and macromolecules [1–7]. A typical FlFFF channel has a rectangular design, with the channel volume which is cut out of a thin ribbon-like spacer clamped with two flat plastic blocks that are embedded with permeable frits on each side (only one side for asymmetrical FlFFF) for the penetration of crossflow [8–10]. HF FlFFF channel uses instead a hollow-fiber membrane as a cylindrical channel. When flow is applied to the hollow fiber, a part of the flow (radial flow) exits through the permeable wall of the fiber membrane, and the remainder (axial flow) sweeps out the end of the fiber. Separation in HF FlFFF is achieved by controlling the radial flow rate which plays the role of a driving force to differently retain sample components within the fiber toward the fiber outlet. When particles or macromolecules are exposed to the field (radial flow movement) in the HF channel, they reach an equilibrium distance that is located slightly away from the fiber wall by the counter-balance of the diffusion of sample components and the field. Sample components of smaller size or lower MW are located at more elevated, average equilibrium position than those of larger size or higher MW and, thus, they will sweep out the HF channel at higher migration velocities due to the parabolic properties of the mobile phase flow movement in the axial direction [1]. Therefore, separation is achieved in the increasing order of particle sizes or MW like in the case of rectangular flow FFF channel systems.

HF FlFFF has gained increasing interest since the HF channel module is simple and inexpensive to assemble, and thus potentially disposable. In addition, HF FlFFF has recently shown that its separation resolution was comparable to that of rectangular type of FlFFF [4]. Earlier studies of HF FlFFF were focused on the use of HF as a channel for FlFFF [1, 2, 11]; the potential of the new technique was enlarged with applications to particles, cells, bacteria, and synthetic organic-soluble polymers [12–17]. Recently, an attempt has been made to interface HF FlFFF with ESI/MS online for protein separation and simultaneous characterization [7].

While HF FlFFF provides a number of advantages mentioned above, it still needs to be optimized by a systematic examination of all the possible factors improving fractionation performance, including resolution, reproducibility, *etc.* In this study, we have evaluated the effects of flow rate conditions and fiber length on protein separation effi-

Correspondence: Dr. Myeong Hee Moon, Department of Chemistry, Yonsei University, Seoul 120-749, Korea.
Fax: +82-2-364-7050. E-mail: mhmoon@yonsei.ac.kr.

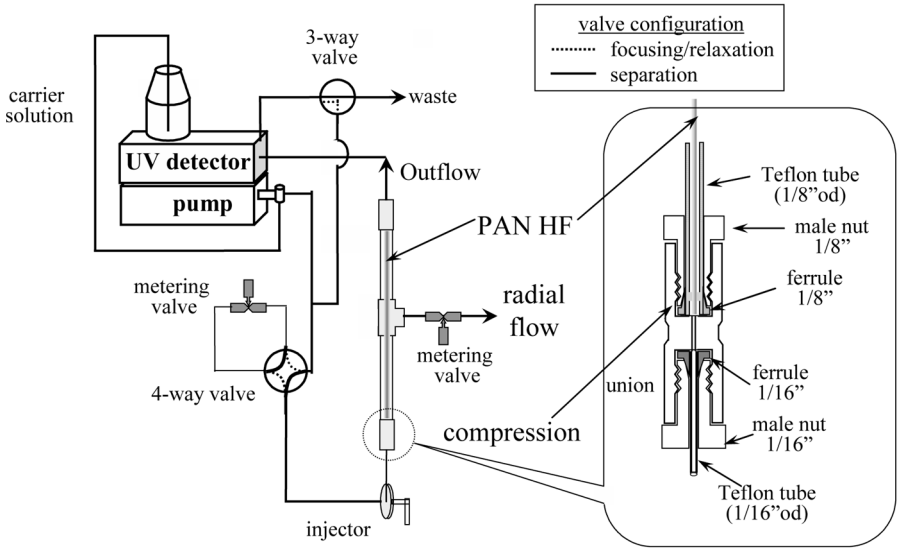

Figure 1. HF FIFFF system scheme and enlarged view of the connection between the HF module, tubings, and union. Both valves (three- and four-way) are oriented as dotted line configuration during focusing/relaxation, and then they are resumed to the solid line configuration for the separation mode.

ciency. Interfiber reproducibility was also evaluated by measuring variations in retention times, peak recovery values, and plate heights. Following this approach, HF FIFFF was then applied to the separation and selective detection of stained lipoproteins contained in human blood plasma samples from healthy persons, and from patients having coronary artery disease (CAD).

2 Experimental

The HF FIFFF channel module was prepared in our laboratory. The HF material used in this work was polyacrylonitrile (PAN) having a dimension of 1.0 mm × 1.4 mm (ID × OD) with a molecular weight cutoff of 30 000 from Chemicore (Daejeon, Korea). The length of HF varied from 10 to 47 cm. The HF FIFFF module was made by inserting HF into a 3.2 mm-OD Teflon tubing (1.6 mm-ID), and connecting it with Teflon tubings without using glue. Since the outer diameter of HF was slightly smaller than the inner diameter of the HF module, the connection between the fiber module and Teflon tubing (1.6 mm-OD and 0.254 mm ID) leading to and from the fiber module was made by a compression using 1/8-in. hand-tight ferrules as illustrated in Fig. 1.

HF FIFFF separation of proteins was carried out with a Model 1050 HPLC system equipped with a pump and a photodiode detector from Agilent Technologies (Palo Alto, CA, USA). Carrier solution for HF FIFFF operation was 10 mM NH_4HCO_3 solution prepared from ultrapure water (>18 MΩ) and filtered with membrane filter (pore size: 0.45 μm) before using. Protein standards used in this study were BSA (66 kDa) and apoferritin (444 kDa) from Sigma (St. Louis, MO, USA). Injection amounts were in the range of 2.5 μg for each protein standard using a loop injector having a fixed volume of 20 μL. Human blood plasma samples from healthy donors and patients having CAD proven angiographically were obtained from Seoul National University Hospital (Seoul, Korea). Staining of lipoproteins contained in plasma samples was made by mixing 200 μL of raw plasma sample with 7 μL of 1% Sudan Black B (SBB) contained in DMSO. Sample injection was made at least 40 min after mixing. The mixture (plasma with SBB solution) was diluted to 1 mL in total volume with the carrier solution of HF FIFFF and about 3 μL of the diluted plasma soluition was injected to HF FIFFF separation.

HF FIFFF operations were carried out by the following steps: (a) sample injection while focusing flows are introduced from both ends of the fiber and (b) elution. At the focusing/relaxation step, the pump flow was divided into two parts (1 : 9 ratio) using the metering valve shown in Fig. 1: One part enters the fiber inlet at one-tenth of the total flow rate, and the rest (9/10) was made to enter through the fiber outlet. During the focusing/relaxation step, the configuration of both the four- and the three-way valves was simultaneously positioned with a dotted line configuration in Fig. 1. After sample injection which was followed by focusing/relaxation for a certain period of time to assure that sample components were expected to reach equilibrium, both valve configurations were switched back to the solid line connection (as they appear in Fig. 1) so that the flow entered only through the inlet of HF. Then the separation of lipoproteins began. The position at which focusing/relaxation occurred at every flow rate condition was visually checked by injecting dye onto HF FIFFF channel. Radial flow rate was kept the same throughout the focusing/relaxation and run modes.

3 Results and discussion

Similar to the conventional rectangular flow FFF techniques, resolution and separation speed in HF FIFFF are

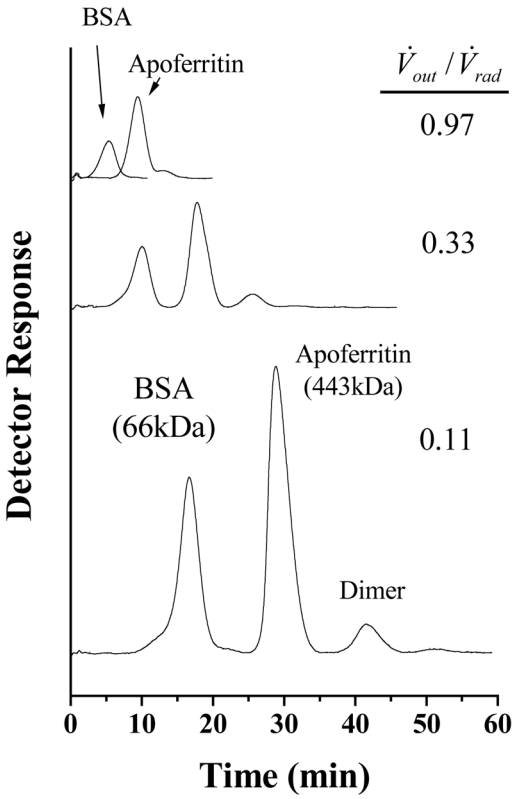

Figure 2. HF FlFFF fractograms of BSA and apoferritin obtained by varying the $\dot{V}_{out}/\dot{V}_{rad}$. Total incoming flow rate was fixed at $\dot{V}_{in}$ = 0.61 mL/min and the outflow rates, $\dot{V}_{out}$, from the top to bottom were 0.30, 0.15, and 0.060 mL/min.

largely influenced by the proper selection of flow rate condition that is obtained by a proper balance between radial flow and outflow rates, and also by the selection of total flow rate. When using HF FlFFF channels, however, a limitation occurs in the maximum value of radial flow rate that can be applied, due to the HF membrane deformability or pore bursting. First of all, we evaluated the efficiency of HF FlFFF separation of protein standards by measuring the experimental plate height values that were obtained by varying the ratio of the outflow rate to the radial flow rate, $\dot{V}_{out}/\dot{V}_{rad}$. Figure 2 shows the separation of BSA (66 kDa) and apoferritin (443 kDa) obtained at a fixed channel inlet flow rate $\dot{V}_{in}$ = 0.61 mL/min, by varying $\dot{V}_{out}/\dot{V}_{rad}$ (except the case corresponding to the top fractograms in Fig. 2, which were obtained for the individual proteins). The length of the HF used for the fractograms in Fig. 2 was 46 cm. As the $\dot{V}_{out}/\dot{V}_{rad}$ ratio decreased, significant improvements in the protein separation were observed. Baseline resolution of the apoferritin aggregates was however obtained at the expense of separation speed. The increase of the signal intensities from the top to bottom fractogram was due to the decrease of the outflow rate from 0.30 to 0.06 mL/min. The experimental plate height values, H_{exp}, obtained at five different run conditions were measured and listed in Table 1 as an average value of triplicate measurements and they are plotted in Fig. 3a. It appears that the H_{exp} was minimized when $\dot{V}_{out}/\dot{V}_{rad}$ decreased to about 0.1: In the case of apoferritin

Table 1. Experimental plate height values (H_{exp}) measured at different $\dot{V}_{out}/\dot{V}_{rad}$. All runs were obtained at a fixed rate of $\dot{V}_{in}$ = 0.61 mL/min with HF length of 47 cm

$\dot{V}_{out}/\dot{V}_{rad}$	H_{exp}, cm	
	BSA	Apoferritin
0.09	1.08 ± 0.06	0.50 ± 0.02
0.11	1.13 ± 0.03	0.43 ± 0.01
0.19	1.48 ± 0.03	0.53 ± 0.02
0.33	2.32 ± 0.03	0.68 ± 0.05
0.97	6.18 ± 0.50	1.98 ± 0.08

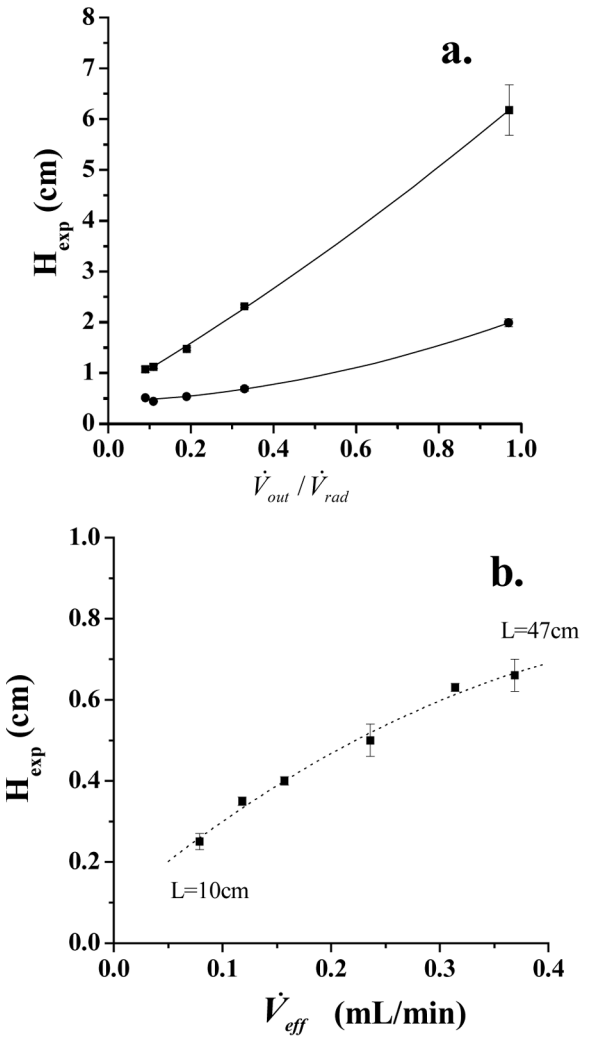

Figure 3. (a) Experimental plate height values of BSA (the upper plot) and apoferritin (the lower one) plotted at various $\dot{V}_{out}/\dot{V}_{rad}$ and (b) plate heights of BSA *versus* effective channel flow rate ($\dot{V}_{ff}$) at various channel lengths.

Table 2. Interfiber reproducibility in terms of retention time (t_r), plate height (H_{exp}), and sample recovery values. All runs were made at the same HF length of 47 cm and $\dot{V}_{in}$ = 0.61 mL/min, $\dot{V}_{rad}$ = 0.55 mL/min

Fiber no.	t_r, min		H_{exp}, cm		Peak recovery, %	
	BSA	Apoferritin	BSA	Apoferritin	BSA	Apoferritin
I	16.3 ± 0.3	28.4 ± 0.4	1.13 ± 0.03	0.43 ± 0.01	80.7 ± 8.7	65.0 ± 2.7
II	16.5 ± 0.2	29.1 ± 0.4	1.59 ± 0.10	0.60 ± 0.04	71.1 ± 2.8	55.8 ± 0.5
III	15.9 ± 0.2	28.4 ± 0.3	1.37 ± 0.05	0.63 ± 0.01	80.9 ± 7.6	65.6 ± 1.8
Average	16.2 ± 0.4	28.6 ± 0.6	1.36 ± 0.12	0.55 ± 0.04	77.6 ± 11.9	62.1 ± 3.3

the number of plates was calculated to be N = 96. Resolution achieved in the fractograms of Fig. 2 indicates that the HF FlFFF performance for protein samples is comparable to that which can be obtained using a conventional, flat-channel FlFFF channel [18], except the low separation speed in HF FlFFF. Since $\dot{V}_{rad}$ = 0.54 mL/min used in the case of the bottom run of Fig. 2 resulted to be the highest radial flow rate value that did not induce HF membrane bursting, the use of a higher radial flow rate in order to enhance resolution in the case of separating a broad MW range of proteins as shown in Fig. 2 was not available.

As long as the performance and applications of HF FlFFF technique increase, the interfiber reproducibility has become one of the major concerns, due to the possible variation in HF membrane properties such as surface smoothness, uniformity of pores, inner diameters, *etc*. Experiments were carried out with three different fibers consecutively using the same run conditions used to obtain the bottom fractogram in Fig. 2. Average data of retention time, plate height, and peak recovery for the two protein samples are listed in Table 2. The relative difference of retention times for the three different fibers (triplicate measurements with each fiber) was found to be less than 3% for both protein samples. This variability is quite acceptable. The PAN HF membrane used in this study appeared to have a relatively high consistency, while polysulfone (PSf) HF membranes used in the previous works had given a relatively higher interfiber variation (data not shown). The variation in plate height measurements observed in this work for PAN HF membranes however appeared to be about 8%, which was somewhat higher than that observed for the retention time measurements. Since the HF FlFFF channels were built without using glue, variation in fractogram broadening due to the difference in the dead volumes at or around tubing connections can be minimized or, at least, be reproducible. A possible variation in fractogram broadening can in fact arise from nonuniformity of the HF pore sizes or to a different surface roughness of the inner membrane wall.

HF FlFFF performance and reproducibility should also be evaluated in terms of sample recovery. The variation in sample recovery observed within three different HF FlFFF channels was about 15% for the lower MW protein (BSA) sample, whereas it was rather smaller (~5%) for the larger MW protein (apoferritin). However, the average recovery value for the longer retained sample (apoferritin, ~62%) appeared to be lower than that for the shorter retained sample (BSA, ~78%). Possible sample loss due to sample penetration through the HF membrane pores could be a source of reduction in sample recovery. However, sample penetration into HF pores should be enlarged when increasing the radial flow rate, and then it would more likely influence the recovery of BSA, the MW of which is close to the MW cutoff (30 kDa) of the PAN HF used in this study. In fact, since MW of apoferritin is higher than that of BSA, it is expected to migrate at an equilibrium height that is closer to the HF inner wall than BSA. Apoferritin then has more chance to experience unwanted interactions with the inner HF membrane wall, which eventually resulted in protein adsorption and recovery reduction of apoferritin with respect to BSA. Because the two contributions can play opposite effects on the recovery of BSA and apoferritin, a systematic approach is needed to evaluate the most predominant contribution to sample recovery.

When considering surface interaction as a source of sample loss, it is known that surface interaction may occur during the focusing/relaxation period or the elution. In order to evaluate the degree of the sample adsorption on the HF inner wall, the effect of length variation of HF fiber was considered. Experiments were carried out by measuring sample recovery using six different HF lengths. Flow rate values were adjusted so that each run was performed with an HF channel of a different length to provide an equivalent field strength (or mean radial flow velocity at the fiber wall), and void time. Table 3 lists the observed retention time, sample recovery, and experimental plate height values for the BSA fractograms obtained at six different HF channel lengths. The average void time for all conditions was 1.19 ± 0.03 min, and the average radial flow velocity at the fiber wall was 0.11 ± 0.002 cm/s. Observed retention time values for each case varied within ~2%, which was quite acceptable, and the RSD for all data was 4%. Since these experiments were performed to examine the effect of channel length on sample recovery by reducing the length of a single 47-cm-long HF fiber from both

Table 3. Effect of HF length on BSA retention. All flow rate conditions were adjusted to have an equivalent field strength and retention time. Average void time calculated from all run conditions is 1.19 ± 0.03 min

Fiber length, cm	$\dot{V}_{out}$, mL/min	$\dot{V}_{rad}$, mL/min	Calculated, t^0, min	t_r, min	Recovery, %	H_{exp}, cm
47	0.081	0.72	1.18	15.7 ± 0.3	77.6 ± 11.9	0.66 ± 0.04
40	0.71	0.61	1.15	14.6 ± 0.1	75.0 ± 7.7	0.63 ± 0.01
30	0.051	0.47	1.19	15.4 ± 0.3	78.1 ± 8.2	0.50 ± 0.04
20	0.035	0.31	1.18	15.2 ± 0.2	75.0 ± 7.7	0.40 ± 0.01
15	0.026	0.23	1.20	16.4 ± 0.2	78.3 ± 8.1	0.35 ± 0.01
10	0.016	0.15	1.25	15.7 ± 0.2	65.6 ± 6.8	0.25 ± 0.02

ends to provide shorter HF lengths, the possibility of sample loss caused by interfiber differences in surface property or irregularity of pores when using different fibers was excluded. This allowed us to eliminate the zone of the HF membrane where focusing/relaxation of the sample occurred for each run at different HF length. Thus, it avoided possible adsorption of proteins above the possibly adsorbed ones left over in previous runs at the focusing/relaxation zone. Table 3 shows that the average recovery values measured with each HF length are comparable, with the exception of the 10-cm-long HF case. It supports that sample loss in HF FIFFF operation may originate from different factors other than the HF fiber length. It meant that sample loss during migration was not critical and much can be referred from during the focusing/relaxation process. While it was reported in a recent study, using a PSf HF membrane, that a quite uniform protein layer was found all along the fiber rather than in the focusing zone [18], the current study did not show the same trend according to the observed data for different lengths of HF fibers. This may originate from the difference in the hydrophobicity of membrane materials (PAN HF membrane is more hydrophobic than PSf). It cannot exclude that some perturbation might occur to sample components from their equilibrium states due to the abrupt change of flow direction when the focusing flow was directed to the inlet of fiber, and then they eluted with void peak or were diluted. Altogether, the experiments cannot surely assess that the focusing/relaxation process was a primary contributor to sample loss. However, the average recovery values reported in Table 3 are about 10% lower than those obtained by a frit inlet asymmetrical FIFFF (FI AFIFFF) channel system, which utilizes hydrodynamic relaxation technique in which relaxation is achieved without focusing [17]. A direct comparison of sample recovery values requires a more systematic study since there is a difference between the membrane materials used in both systems. A final evaluation of the HF FIFFF performance in protein separation can be given by observing the effect of HF length on the efficiency. When the plate height values measured for the different lengths are compared, it was observed that plate height decreased with decreasing fiber length. It must be noted that while the channel void time and field strength were adjusted to be identical at each HF length, the effective channel flow rate (equivalent to the channel flow rate of a symmetrical rectangular channel system) in fact decreased with decreasing HF length. As a consequence, the observed decrease of plate height with decreasing HF length demonstrates that band broadening from nonequilibrium phenomena in HF FIFFF decreased when the effective channel flow rate decreased as shown in Fig. 3b. Similar to the flow patterns in an asymmetrical flow FFF, the effective channel flow rate, $\dot{V}_{eff}$, of HF FIFFF is dependent on the radial flow rate (equivalent to cross flow rate) and can be expressed as follows:

$$V_{eff} = \frac{V^0}{t^0} = \dot{V}_{rad}\left(\ln\frac{\dot{V}_{in}}{\dot{V}_{out}}\right)^{-1} \quad (1)$$

where V^0 and t^0 are the void volume and the void time, respectively, and $\dot{V}$ denotes the volumetric flow rate which is used with subscripts of out, in, and rad for outflow, total incoming flow, and radial flow, respectively.

Performance of HF FIFFF for protein fractionation was finally tested with a real protein sample. Lipoproteins contained in human blood plasma from healthy donors and from patients with CAD were considered. In an earlier study, FI AFIFFF was applied for identifying the decrease of particle size of low density lipoproteins (LDL) as well as the decrease of amount of high density lipoproteins (HDL) in blood plasma from patients with CAD [19]. It therein highlighted the potential application of FIFFF for clinical diagnosis by calculating LDL sizes from the FIFFF elution peak through a direct injection of plasma. However, in FI AFIFFF run-to-run sample carryover due to incomplete sample recovery is possible, as shown in the sample recovery study discussed above. However, simplicity of HF FIFFF channel and a low cost of the HF membrane reducing these drawbacks make it possible for a disposable use of the channels. Figure 4 shows the separation of lipoproteins of plasma samples from a healthy donor and from a patient with CAD. Fractograms were obtained at

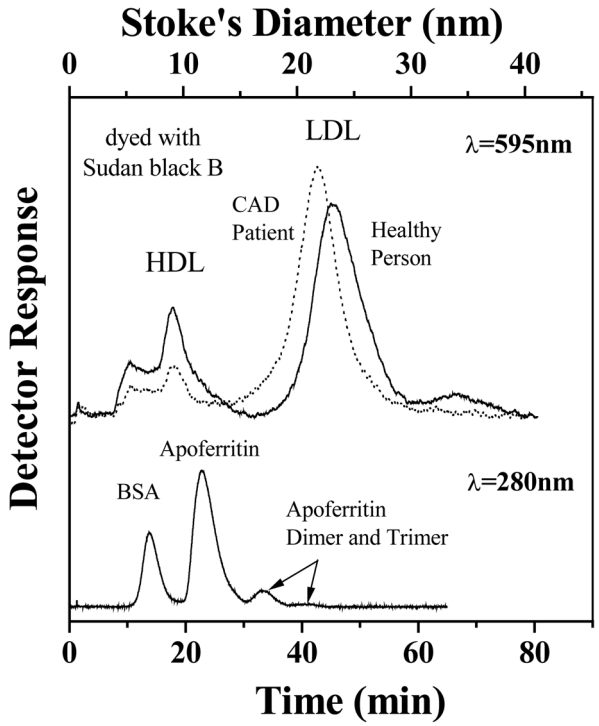

Figure 4. HF FlFFF of lipoproteins from a healthy donor and from a patient with CAD, along with the fractogram of protein standards. All runs were performed at $\dot{V}_{out}$ = 0.03 and $\dot{V}_{rad}$ = 0.30 mL/min.

$\dot{V}_{out}$ = 0.03 and $\dot{V}_{rad}$ = 0.30 mL/min, with an HF length of 26 cm. Sample injection was made with 3 μL of stained plasma sample which was previously diluted five times. Thus, the volume of the net serum sample injected was nearly 0.6 μL. Plasma samples were stained prior to injection with Sudan Black B, which is a specific dye for lipid components in lipoproteins, and detection was made at 595 nm. In the earlier work [19], direct monitoring of the lipoproteins at 280 nm without staining was proven to be nonselective because of the interference of high-abundance serum proteins. In the upper fractograms of Fig. 4, the lipoprotein fractogram of the sample from a CAD patient showed a clear shift in the peak maximum corresponding to fractionated LDL compared to the fractogram of a healthy donor. By applying the HF FlFFF retention theory [4], the retention time scale can be converted into Stokes' diameter scale (upper horizontal axis in Fig. 4) using the following equation:

$$d_s = \frac{8kTt_r}{3\pi\eta r_f^2} \ln\left(\frac{\dot{V}_{out}}{\dot{V}_{in} - (L_0/L)\dot{V}_{rad}}\right) \quad (2)$$

where kT is thermal energy, t_r the retention time, η the mobile phase viscosity, r_f the inner radius of the HF, L the HF length, L_0 the distance between the focusing point and the HF outlet. According to Eq. (2), the Stokes' diameter of LDL from the CAD patient sample, determined in correspondence of the retention time of the LDL fractogram maximum, was calculated to be 21.8 nm. This size value is in very good agreement with the size value (21.6 nm) observed in the earlier report on FI AFlFFF of lipoproteins [19]. The Stokes' diameter of LDL determined from the healthy donor person was 23.2 nm. There is a possibility of variation in the retention of lipoproteins among different healthy patients (or among CAD patients), depending on their cholesterol levels as shown in an earlier work. However, the relative amount of the secondary peak of HDL with CAD was greatly decreased while the particle size of HDL did not appear to change, in which the latter was consistent with the fact that low levels of HDL cholesterol have a strong association with CAD in patients.

4 Concluding remarks

In this study, performance of HF FlFFF separation was examined first with protein standards and, then, applied with human lipoprotein samples. With standard proteins it was found that most efficient protein separation can be obtained at a small $\dot{V}_{out}/\dot{V}_{rad}$ ratio (1:9), and the sample recovery was affected by protein adsorption on the HF inner wall. While HF FlFFF of proteins in a broad M-range is limited by the selection of a maximum radial flow rate that can be used without damaging the HF membrane, it is proved that HF FlFFF can provide a fractionation performance that is comparable to that obtained through conventional, flat-channel FlFFF systems. Possible disposable usage of the HF FlFFF channel makes the more feasible application to samples of biological or clinical interest, being avoided run-to-run sample carryover and being reduced sterility issues. However, as for possible concerns on interfiber reproducibility, HF membranes used in this study showed a relatively small variation in retention time and sample recovery, though they exhibited significant differences in efficiency. This would imply a possible variation in membrane properties among different fibers. The higher reproducibility observed among PAN fibers can be ascribed to a higher membrane rigidity with respect to PSf or PVC HF used in earlier studies.

HF FlFFF performance in the fractionation of real protein samples was shown using less than 1 μL of blood plasma. HF FlFFF successfully fractionated lipoproteins according to differences in Stokes' size. In addition, it demonstrated that HF FlFFF can be utilized to evaluate size reduction of the LDL fraction present in serum samples taken from patients with CAD.

This study was supported by a grant from Korea Ministry of Science & Technology (MOST) in 2004 and partly by the Maeji Research Fund provided by Yonsei University in 2003. The authors acknowledge the support of an HPLC system for this study from the Advanced Analysis Center

at KIST. Thanks are also given to P. Reschiglian, University of Bologna, for his fruitful discussion.

5 References

[1] Jönsson, J. A., Carlshaf, A., *Anal. Chem.* 1989, *61*, 11.

[2] Carlshaf, A., Jönsson, J. A., *J. Chromatogr.* 1989, *461*, 89.

[3] Wijnhoven, J. E. G. J., Koorn, J.-P., Pope, H., Kok, W. Th., *J. Chromatogr.* 1995, *699*, 119.

[4] Lee, W. J., Min, B.-R., Moon, M. H., *Anal. Chem.* 1999, *71*, 3446.

[5] Bruijinsvort, M. V., Kok, W. T., Tijssen, R., *Anal. Chem.* 2001, *73*, 4736.

[6] Reschiglian, P., Zattoni, A., Cinque, L., Roda, B., Piaz, F. D., Roda, A., Moon, M. H., Min, B. R., *Anal. Chem.* 2004, *76*, 2103.

[7] Reschiglian, P., Zattoni, A., Roda, B., Cinque, L., Parisi, D., Roda, A., Piaz, F. D. *et al.*, *Anal. Chem.* 2005, *77*, 47.

[8] Yang, F. J. F., Myers, M. N., Giddings, J. C., *Anal. Chem.* 1974, *46*, 1924.

[9] Giddings, J. C., *Science*, 1993, *260*, 1456.

[10] Wahlund, K.-G., Giddings, J. C., *Anal. Chem.* 1987, *59*, 1332.

[11] Jönsson, J. A., Carlshaf, A., *J. Microcol. Sep.* 1991, *3*, 411.

[12] Wijnhoven, J. E. G. J., Koorn, J.-P., Pope, H., Kok, W. Th., *J. Chromatogr.* 1996, *732*, 307.

[13] Moon, M. H., Lee, K. H., Min, B. R., *J. Microcol. Sep.* 1999, *11*, 676.

[14] Reschiglian, P., Roda, B., Zattoni, A., Min, B. R., Moon, M. H., *J. Sep. Sci.* 2002, *25*, 490.

[15] Min, B. R., Kim, S. J., Ahn, K.-H., Moon, M. H., *J. Chromatogr. A* 2002, *950*, 175.

[16] Reschiglian, P., Zattoni, A., Roda, B., Cinque, L., Mellucci, D., Min, B. R., Moon, M. H., *J. Chromatogr. A* 2003, *985*, 519.

[17] Moon, M. H., Hwang, I., *J. Liq. Chromatogr. Relat. Technol.* 2001, *24(20)*, 3069.

[18] Roda, B., Cioffi, N., Ditaranto, N., Zattoni, A., Casolari, S., Melucci, D., Reschiglian, P. *et al.*, *Anal. Bioanal. Chem.* 2005, *381*, 639.

[19] Park, I., Paeng, K.-J., Yoon, Y., Song, J.-H., Moon, M. H., *J. Chromatogr. B* 2002, *780*, 415.

