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Original Paper

Miniaturized asymmetrical flow field-flow fractionation: Application to biological vesicles

Asymmetrical flow field-flow fractionation (AFlFFF) has been carried out in a miniaturized channel by reducing the channel dimensions. Performance of the miniaturized AFlFFF (mAFlFFF) channel was evaluated with standard proteins and polystyrene latex spheres from nanometer to micrometer size. By reducing the channel dimension, proteins or particulate materials can be separated within a few minutes without a significant loss in resolution. The mAFlFFF channel was applied for the separation of exosomes harvested from immortalized human mesenchymal stem cell line. It shows a potential to fractionate exosome vesicles according to sizes which can be useful for proteomic studies in relation to immunotherapeutic applications.

Keywords: Asymmetrical flow field-flow fractionation / Exosome / FFF / Miniaturized FFF channel / Protein separation

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1 Introduction

Flow field-flow fractionation (FlFFF) has been widely utilized for the separation and characterization of macromolecular species in an empty channel space [1 – 6]. Since retention of sample components in field-flow fractionation (FFF) is normally achieved by the application of an external field to the direction perpendicular to the migration flow (or channel flow), separation in FlFFF is obtained by controlling the crossflow rate which moves across the channel and plays a role of external field. In an asymmetrical FlFFF (AFlFFF) channel equipped with only one permeable wall, flow entering the channel inlet is divided into two parts so that a part of the incoming flow penetrates the channel wall (crossflow) and the rest exits the channel outlet toward the detector (outlet flow) [3, 4, 7]. Thus, AFlFFF separation is governed by the manipulation of both crossflow and outflow rates.

When applied to the AFlFFF channel, crossflow drives sample components toward the accumulation wall, which is counterbalanced by the diffusion of sample molecules acting against the channel wall. With the application of crossflow, sample materials are vertically distributed at the vicinity of the channel wall according to their diffusion coefficients. When migration flow is applied, small MW components with relatively large diffusion coefficients migrate along the channel faster than large MW components with relatively small diffusion coefficients due to the parabolic nature of laminar flow in a thin FFF channel. Thus, separation in FlFFF is achieved in an order of increasing MW or hydrodynamic diameter of the analyte.

In practice, AFlFFF requires the focusing/relaxation process to assure equilibrium distribution of sample components above the channel wall by the balance of an external field force and diffusion. This is achieved in such a way that the two counterdirected flow streams (one from the channel inlet and the other from the channel outlet) are adjusted to converge at an injection point near the channel inlet. During a certain period of focusing/relaxation process in AFlFFF, initial sample bandwidth can be minimized. Because its resolution is superior to other FFF subtechniques, AFlFFF has been widely utilized for the separation of proteins, water soluble polymers, and particulate materials [8 – 13].

Miniaturization of separation techniques is generally advantageous in reducing injection amount and mobile phase consumption as well as in enhancing separation speed and resolution [14]. Miniaturization of FlFFF was first attempted with a frit inlet asymmetrical FlFFF (FI-AFlFFF) [15] and recently applied to AFlFFF [16]. FI-AFlFFF is a modified AFlFFF channel which is designed to bypass the focusing/relaxation procedure by utilizing a high speed flow stream through a small inlet frit nearby the channel inlet of the depletion wall of AFlFFF channel [17 – 19]. Utilization of microbore hollow fiber as an FlFFF channel made it possible to manipulate the hollow fiber FlFFF (or HF FlFFF) operated at few μL/min regime of out-

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flow rate for protein separation. The microbore HF FlFFF technique was then integrated toward the development of online 2-D protein separation technique with the hyphenation of CIEF, and the developed online CIEF-HF FlFFF was applied for the fractionation of human urinary proteome based on p*I* and MW followed by nanoflow LC-MS/MS for proteomic analysis [20].

In this study, a miniaturized AFlFFF channel was built by reducing the conventional channel dimension to achieve high speed separation without increasing the migration flow rate. While the miniaturized AFlFFF channel attempted first by Yohannes *et al.* [16] utilized a channel thickness of 500 μm, the current study demonstrated the use of a much thinner channel (178 μm) for high speed separation together with an increase in experimental plate number. The separation efficiency of the method is examined using proteins and nanometer to micrometer sized particle standards. The miniaturized AFlFFF channel was applied for the separation of exosomes, small membrane vesicles (less than 100 nm in diameter) secreted from cells as a consequence of fusion of multivesicular late endosomes/lysosomes with plasma membrane [21, 22].

2 Experimental

A miniaturized AFlFFF channel was built in our laboratory. The channel was assembled similarly to the construction of the miniaturized FI-AFlFFF channel. The two lucite blocks were cut into an exterior dimension of $13 \times 4.5 \times 2$ cm^3 (length × width × height). A ceramic frit ($10 \times 1.5 \times 5$ cm^3) was embedded in only one channel block. The other channel block was used without frit. For channel space, it was made by cutting a Mylar sheet in a ribbon-like shape. Two channels were made using 178 (named as channel I) and 254 μm (channel II) thick Mylar sheets. Each channel was cut into a trapezoidal shape having a tip-to-tip length of 9.3 cm with an initial breadth of 0.7 cm and a final breadth of 0.3 cm. Both ends of the trapezoidal channel space were cut into triangular shape having lengths of 0.7 and 0.3 cm for the inlet and outlet ends, respectively. Geometrical channel volumes were 79 and 113 μL for the channels I and II, respectively. Sample injection was made through an injection port drilled at a distance of 1.0 cm from the channel inlet hole at the depletion channel wall, the plain channel block side. All the flow connections to and from the channel inlet, injection port, and channel outlet were made with Teflon tubings (254-μm id, 1.6-mm od). The dead volume from the channel outlet to the detector was 15 μL.

At the accumulation wall (the channel block with ceramic frit), a PLCGC sheet membrane (MWCO: 10 kDa) from Millipore (Danvers, MA, USA) was placed above the frit to avoid sample loss. As carrier solutions, 0.1 M PBS buffer adjusted at pH 7.4 was used for protein/exosome separation and 0.1% FL-70 mixed with 0.02% NaN_3 was used only for polystyrene (PS) standard latex spheres from Duke Scientific (Palo Alto, CA, USA). All the carrier solutions were prepared from ultrapure water (>18 MΩ), and filtered through a membrane filter with a pore size of 0.45 μm, prior to use. Carrier solution and sample suspension were separately delivered by two identical HPLC pumps, Model 930 from Young-Lin (Seoul, Korea). A metering valve, model Whitey SS-22RS2 from Crawford Fitting Co. (Solon, OH, USA), was located downstream of the detector to provide adequate back pressure and to regulate flow rates. Eluted sample components were detected with a Model 730 UV detector (cell volume: 5 μL) from Young-Lin at a wavelength of 280 nm for proteins and 254 nm for PS.

Standard proteins were carbonic anhydrase (29 kDa), BSA (66 kDa), and alcohol dehydrogenase (150 kDa) from Sigma (St. Louis, MO, USA). PS standard latex spheres were 50, 93, 135 nm, 2.002, 3.004, 4.000, 4.991 μm in nominal diameter from Duke Scientific.

Exosomes were harvested from the immortalized human mesenchymal stem cell line (B10) which was isolated from bone marrow. B10 cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 5% horse serum, and 50 μg/mL gentamicin at 37°C and 5% CO_2. When cells reached ~60% confluence, the medium was changed, and supernatants were collected after a period of 24 h. Supernatants were centrifuged at room temperature at $500 \times g$ for 10 min to remove any dead cell, and subjected to filtration using 0.22 μm filters from Millipore to remove cell debris and large vesicles. Then, exosomes were concentrated using Amicon ultra centrifugal filter devices (MWCO: 65 kDA) from Millipore.

3 Results and discussion

The miniaturized AFlFFF (mAFlFFF) channel was evaluated with standard materials including proteins and nano- to micron-sized latex spheres. Figure 1 shows the high speed separation of protein standard mixtures (carbonic anhydrase (CA), 29 kDa; BSA, 66 kDa; and alcohol dehydrogenase (ADH), 150 kDa) using an mAFlFFF channel I at outflow rate of 0.4 mL/min and crossflow rate of 0.6 mL/min. Injection amounts of each protein sample were 0.2 (CA), 0.5 (BSA), and 1.0 μg (ADH). The separation of the three protein standards was achieved within 3.5 min together with an isolation of a dimer peak of ADH together. The presence of dimers was confirmed by checking the relevant MW value from the observed retention time of the dimer peak using a calibration curve that was set up with the logarithm of retention time *ver-*

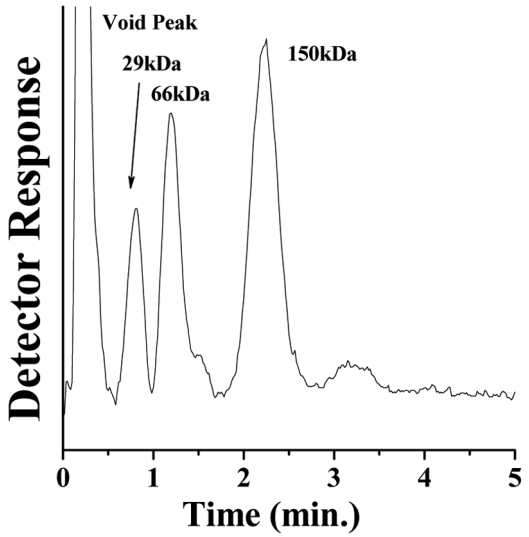

Figure 1. Separation of protein standard mixtures by mAFlFFF obtained at outflow rate ($\dot{V}_{out}$) of 0.4 mL/min and total inlet flow rate ($\dot{V}_c$) of 1.0 mL/min (focusing/relaxation time: 20 s).

sus logarithm of MW (log tr = 0.60(±0.04) log MW-0.98(±0.08), r^2 = 0.990). The result shown in Fig. 1 is faster than what can be obtained from the miniaturized frit inlet AFlFFF (mFI-AFlFFF). FI-AFIFF requires a reduced outflow rate since separation is achieved continuously by hydrodynamic relaxation without stopping the migration flow, which makes it necessary to keep the ratio of outlet flow to crossflow rate minimum for maintaining a sample band without being broadened. Since equilibrium of sample components prior to separation in AFlFFF channel can be well established by the focusing/relaxation process, separation in AFlFFF channel is more flexible than FI-AFlFFF channel in selecting outlet flow rate condition to increase the speed of separation.

Technical performance of the mAFlFFF channel was examined by measuring experimental plate height and the recovery of sample components after separation. Figure 2a shows the elution profiles of BSA obtained by varying outflow rate but with a fixed rate (1.0 mL/min) of a total inlet flow (sum of outflow rate and crossflow rate). As outflow rate ($\dot{V}_{out}$) expressed in Fig. 2a decreases, crossflow rate ($\dot{V}_c$) increases simultaneously and the BSA peak becomes broader together with a clear isolation of dimer peak together. Measured average plate height values (n = 3) are 2.10(±0.39) mm, 0.63(±0.04), 0.43(±0.03), 0.27(±0.01), 0.21(±0.01), and 0.36(±0.08) mm at an order of decreasing outflow rate. It is found that the efficiency of the current mAFlFFF channel is maximized at $\dot{V}_{out}$/$\dot{V}_c$ = 0.10/0.90 mL/min and the plate number of the corresponding peak is calculated as 443. A similar evaluation of mAFlFFF channel was recently reported by Yohannes *et al.* [16] with a maximum plate number of 367 using a

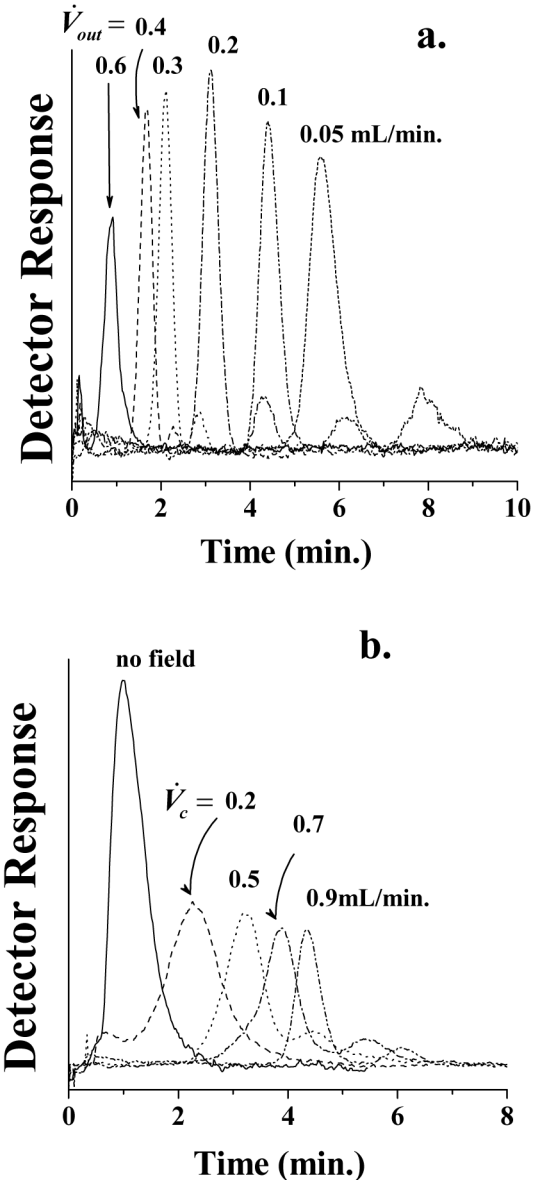

Figure 2. (a) Elution profiles of BSA by varying the outflow rate (but at a fixed $\dot{V}_{in}$ = 1.0 mL/min) for the measurement of experimental plate height values and (b) the effect of sample recovery (sample: BSA) at a fixed $\dot{V}_{out}$ (=0.10 mL/min) by varying crossflow rate ($\dot{V}_c$).

same BSA standard (experimental conditions $\dot{V}_{out}$/$\dot{V}_c$ = 0.05/0.77 mL/min, a trapezoidal channel with 11 cm in length and 500 μm in thickness). The retention time of BSA in our channel I at its highest efficiency was about 4.4 min, whereas that reported in the ref. [20] was 18.3 min. This is due to the difference in the thickness (178 μm for the channel I and ~500 μm for the channel used in the ref. [20]). However, the time-based rate of generation of efficiency, N/t [23], in our experiment is about five times larger (N/t = 100 plates/min) than the calculated value (~20) from the reported plate height since

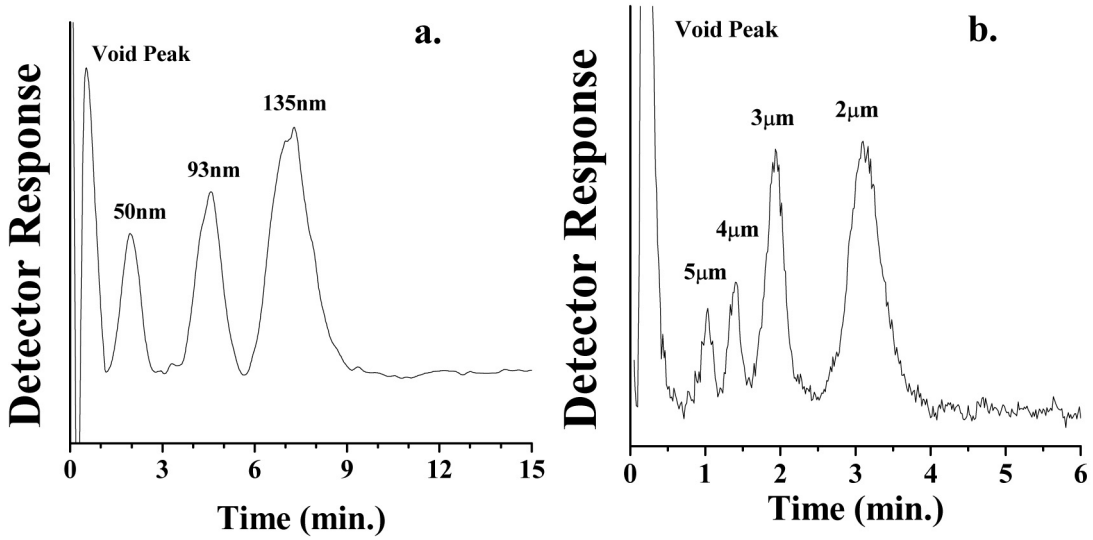

Figure 3. High-speed separation of polystyrene latex spheres in (a) normal mode and (b) hyperlayer mode of FlFFF separation using mAFlFFF channel. Flow rate conditions are (a) $\dot{V}_{out}/\dot{V}_c$ = 1.40/0.10 and (b) $\dot{V}_{out}/\dot{V}_c$ = 0.84/0.16 mL/min.

retention time of our experiment is about four times faster. In this study, since we have utilized 254 μm id Teflon tubing for the connection of channel outlet to the detector, a possibility of postchannel band broadening cannot be overlooked. Since there was a strong back pressure during focusing/relaxation when a smaller diameter tubing such as silica capillary was used in between the channel outlet to the detector, postchannel dead volume (15 μL) could not be minimized any further. This is one of the drawbacks of our current system which may induce a band broadening during the passage of eluting components to detector from the channel outlet. However, the transient time from the channel outlet to detector was calculated to be approximately 5 s for the case of protein/exosome separations and about 1 s for particle separations in later experiments, the postchannel band broadening may not be a serious factor at the flow rate conditions employed here

The plate number observed with mAFlFFF channel is much larger than that reported with microbore HF FlFFF. These differences result from the applicability of higher crossflow rate in mAFlFFF channel. In contrast, HF channel system has a certain limitation to utilize a stronger radial flow rate due to the fowling of membrane fiber. Moreover, it is obviously larger than those of mFI-AFlFFF channel system since the relaxation of sample components can be more efficiently achieved in mAFlFFF during focusing/relaxation process than in mFI-AFlFFF.

Recovery of sample components during mAFlFFF separation is an important issue since sample adsorption may occur at the channel membrane during focusing/relaxation and elution. Figure 2b shows the fractograms of BSA measured by varying the crossflow rate at a fixed outflow rate of 0.10 mL/min. By increasing the crossflow rate, it appears with the increase in retention time. Recovery value was calculated by comparing the peak area obtained at each run condition with that obtained without applying field strength. Recovery values at increasing crossflow rate (0.20, 0.30, 0.50, 0.70, and 0.90 mL/min) were calculated to be 75.2%(±1.9), 60.6%(±1.9), 56.7%(±8.2), 46.9%(±4.4), and 36.7%(±3.6), respectively (n = 3). While mAFlFFF channel system provided a higher separation efficiency as explained above with plate number, sample recovery values appear to be somewhat lower than what can be usually obtained with FI-AFlFFF system (larger than ~80%) that runs without focusing/relaxation [24]. When recovery values of BSA reported by a microbore HF FlFFF channel (450 μm id × 25 cm, polysulfone) were compared with those obtained by the current mAFlFFF channel, they were about 78 – 54% at radial flow rates varying from 0.1 to 0.5 mL/min under a fixed outflow rate of 70 μL/min which were similar to each other. In relation to sample recovery, minimum sample injection amount was examined at a harsh condition of $\dot{V}_{out}/\dot{V}_c$ = 0.18/0.82 mL/min. Experiments were done by varying the injection amount from 500 to 100 ng of BSA and the LOD was calculated as 57 ng (0.86 pmol) from calibration, approximately two times larger than the value reported in microbore HF FlFFF experiment using the same sample.

The miniaturized AFlFFF channel has been tested with the separation of polystyrene latex standards at both normal and hyperlayer mode [25] of separation. Figure 3 shows the two AFlFFF fractograms for the separation of (i) nanosized polystyrene latex particles and (ii) supramicron-sized PS particles obtained at $\dot{V}_{out}/\dot{V}_c$ = 0.84/0.16 and 1.40/0.10 mL/min, respectively, using the channel II (254 μm thick). While PS particles in Fig. 3a were sepa-

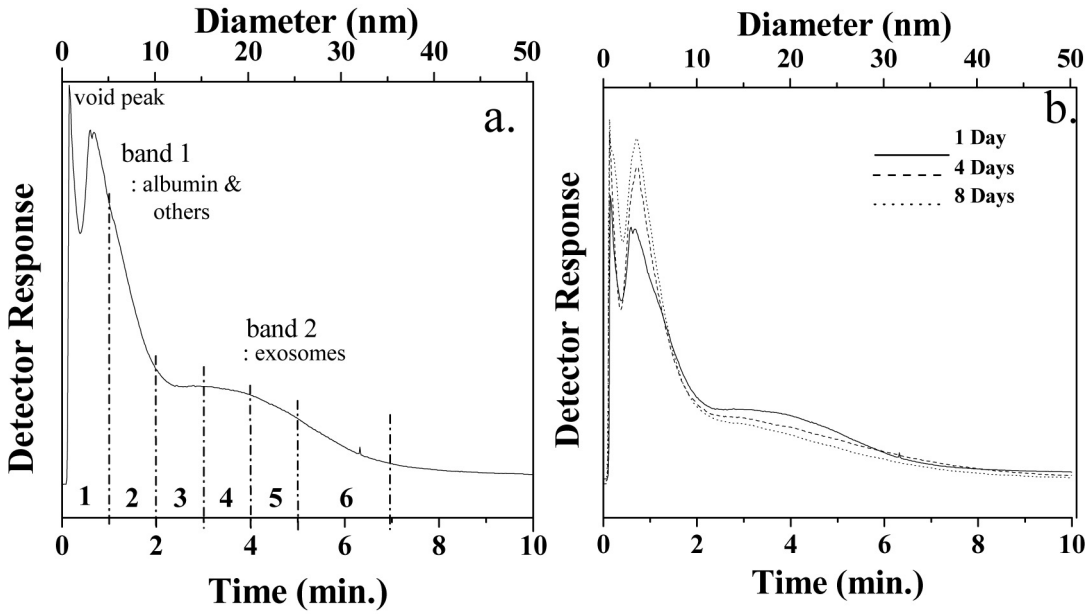

Figure 4. (a) mAFlFFF fractogram of the exosome sample harvested from human mesenchymal stem cell line (B10) obtained at $\dot{V}_{out}/\dot{V}_c$ = 0.17/0.53 mL/min with fractions collected for proteomic analysis and (b) aging effect on the peak intensity of exosome.

rated by an increasing order of particle size (normal mode of FFF retention), Fig. 3b shows a typical steric/hyperlayer separation (particle diameter > ~1 μm) in which elution order is reversed. Figure 3 demonstrates that miniaturized AFlFFF can be utilized for the separation of small biological vesicles or cells. By utilizing FlFFF theory, the effective channel thickness of the channel II was calculated as 217 ± 17 μm from the experimental retention time of each nanoparticle standard.

The mAFlFFF channel I has been applied for the separation of exosomes, which are small membrane vesicles, harvested from the B10 cell line. Although size is an important criterion for the identification of exosomes, it is not easy to characterize and isolate biological vesicles according to their sizes. Heijnen *et al.* [26] used flow cytometry for the analysis of exosome, yet the mean fluorescence intensity remained within background levels, indicating that exosomes were too small to be detected by flow cytometry. Figure 4a shows the fractogram of an exosome sample from B10 cell line utilizing the mAFlFFF channel at a flow rate condition of $\dot{V}_{out}/\dot{V}_c$ = 0.53/0.17 mL/min. It consists of a tall peak followed by a broad shoulder corresponding to the elution of sample components for 10 min. Calculation of the hydrodynamic diameter values of eluting components was made from retention time using the FlFFF theory. The diameter scale was marked at the top of the plot. For the calculation, channel thickness value utilized was as 170 ± 2 μm which was based on the retention time measurement of standard latex beads. According to the fractogram profile, the exosome sample displays as bimodal size distribution. Each collected fraction was subjected to lysis and tryptic digestion followed by nanoflow LC-ESI-MS/MS for proteomic analysis. For nanoflow LC-ESI-MS/MS analysis, a homemade pulled tip capillary LC column (C18) was utilized with a binary gradient separation. Details of the experiments were explained in an earlier work. The band 1 in Fig. 4a was identified to contain albumin as a major protein by nanoflow LC-ESI-MS/MS followed by database search. Since B10 cells were grown in DMEM supplemented with 5% FBS and 5% horse serum, it is not surprising that the exosome samples harvested from B10 cell line contain serum proteins including albumin. The late eluting fractions (fraction number 4–6) are supposed to be originated from the elution of exosome vesicles. As observed in protein separation in Fig. 1, proteins smaller than 150 kDa eluted within 2.5 min. Therefore, eluting components in the band 2 in Fig. 4a are expected to be much larger in their molecular weight if they are not entrapped in exosome vesicles. LC-ESI-MS-MS analysis of the peptide digests of the fraction 5 gave identifications of α2-HS-glycoprotein (39 kDa), apolipoprotein A-I (30 kDa), and plasminogen (91 kDa) which were reported as exosomal proteins in the literature [27]. Compared with the retention time of standard proteins in Fig. 1, the expected retention time of such small MW proteins can be deduced to be less than 2 min if they are free in the sample solution without being entrapped in vesicles. It supports that the band 2 results from the elution of exosome vesicles.

However, identification of the proteins other than albumin in the collected fraction of exosome peak was only partial since the relative concentration of albumin was too large. Though the mAFlFFF separation of exosome vesicles from albumin or other serum proteins is not completely made in this preliminary study, it shows a great potential of characterizing exosome vesicles utilizing FFF if a proper extraction method is developed to deplete serum proteins (especially albumin) from the mixture. While mAFlFFF was utilized to fractionate exosome vesicles according to molecular sizes, it was observed that peak distribution of the exosome sample significantly changed within few days. The aging effect was observed by the change in elution profile of the exosome sample as shown in Fig. 4b. The exosome sample was repeatedly run at 3 and 7 days later at the same run condition used in Fig. 4a. The exosome sample was kept at 4°C once it was defrosted from −80°C. As a result, the first peak (resulting from albumin and a few proteins) increased while the peak intensity of exosome vesicles decreased, those exosome vesicles were destroyed within few days. A study to improve the extraction procedure is ongoing and in the future it will be integrated for the size-dependent analysis of the proteome of exosome vesicles.

4 Concluding remarks

This study demonstrated that using channels of reduced dimensions can fasten AFlFFF separation without losing the separation capability. The approach was applied to AFlFFF for proteins and nano- to micron-sized particles. When mAFlFFF channel was applied for the separation of exosome, it showed a potential to characterize exosome vesicles according to their sizes. Although in this study, in-depth proteomic analysis of exosomes was hampered by the presence of albumin, it seems evident that mAFlFFF can open up for new opportunities for size fractionation of exosome vesicles, which can be further utilized for size-dependent analysis of exosomal proteins.

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5 References

[1] Giddings, J. C., *Science* 1993, *260*, 1456 – 1465.
[2] Schimpf, M. E., Caldwell, K. D., Giddings, J. C., *Field-Flow Fractionation Handbook*, Wiley Interscience, NY 2000.
[3] Wahlund, K.-G., Giddings, J. C., *Anal. Chem.* 1987, *59*, 1332 – 1339.
[4] Reschiglian, P., Zattoni, A., Roda, B., Casolari, S. *et al.*, *Anal. Chem.* 2002, *74*, 4895 – 4904.
[5] Kang, D., Moon, M. H., *Anal. Chem.* 2005, *77*, 4207 – 4212.
[6] Reschglian, P., Zattoni, A., Roda, B., Michelini, E., Roda, A., *Trends Biotechnol.* 2005, *23*, 475 – 483.
[7] Wahlund, K.-G., Litzén, A. J., *J. Chromatogr.* 1989, *461*, 73 – 87.
[8] Wittgren, B., Wahlund, K.-G., *J. Chromatogr. A* 1997, *760*, 205 – 218.
[9] Duval, C., Le Cerf, D., Picton, L., Muller, G., *J. Chromatogr. B* 2001, *753*, 115 – 122.
[10] Nilsson, M., Wahlund, K.-G., Bulow, L., *Biotechnol. Tech.* 1998, *12*, 477 – 480.
[11] Arfvidsson, C., Wahlund, K.-G., *Anal. Biochem.* 2003, *313*, 76 – 85.
[12] Lee, S., Nilsson, P.-O., Nilsson, G. S., Wahlund, K.-G., *J. Chromatogr. A* 2003, *1011*, 111 – 123.
[13] Fraunhofer, W., Winter, G., *Eur. J. Pharm. Biopharm.* 2004, *58*, 369 – 383.
[14] Szumski, M., Buszewski, B., *Crit. Rev. Anal. Chem.* 2002, *32*, 1.
[15] Kang, D., Moon, M. H., *Anal. Chem.* 2004, *76*, 3851 – 3855.
[16] Yohannes, G., Sneck, M., Varjo, S. J. O., Jussila, M. *et al.*, *Anal. Bioanal.* 2006, *354*, 255 – 265.
[17] Moon, M. H., Kwon, H. S., Park, I., *Anal. Chem.* 1997, *69*, 1436 – 1440.
[18] Moon, M. H., Williams, P. S., Kwon, H., *Anal. Chem.* 1999, *71*, 2657 – 2666.
[19] Lee, H., Kim, H., Moon, M. H., *J. Chromatogr. A* 2005, *1089*, 203 – 210.
[20] Kang, D., Moon, M., *Anal. Chem.* 2006, *78*, 5789 – 5798.
[21] Johnston, R. M., Adam, M., Hammond, J. R., Orr, L., Turbide, C., *J. Biol. Chem.* 1987, *262*, 9412 – 9420.
[22] Denzer, K., Kleijmeer, M. J., Heijnen, H. F., Stoorvogel, W., Geuze, H. J., *J. Cell Sci.* 2000, *113*, 3365 – 3374.
[23] Giddings, J. C., *Unified Separation Science*, Wiley Interscience, NY 1991, p. 176.
[24] Moon, M. H., Hwang, I., *J. Liq. Chromatogr. Relat. Technol.* 2001, *24*, 3069 – 3083.
[25] Ratanathanawongs, S. K., Giddings, J. C., *Chromatographia* 1994, *38*, 545 – 554.
[26] Heijnen, H. F., Schiel, A. E., Fijnheer, R., Geuze, H. J., Sixma, J. J., *Blood* 1999, *94*, 3791 – 3799.
[27] Mears, R., Craven, R. A., Hanraham, S., Totty, N., *et al.*, *Proteomics* 2004, *4*, 4019 – 4031.

