



Short communication

Evaluation of multiplexed hollow fiber flow field-flow fractionation for semi-preparative purposes

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ABSTRACT

A multiplexed hollow fiber flow field-flow fractionation (MxHF5) is introduced to increase throughput of an HF5 channel system for semi-preparative purposes. HF5, a cylindrical version of the flow field-flow fractionation (FIFFF) operated with a porous, hollow fiber membrane by controlling the ratio of radial and axial flow rates, is capable of fractionating proteins, cells, and macromolecules by size. An advantage of HF5 is its inexpensive channel construction, allowing for disposability that can reduce run-to-run carryover problems. MxHF5 constructed in this study was made with six parallel HF5 modules connected to seven-port manifolds for the semi-preparative scale separation of proteins or biological particles. For the evaluation of MxHF5 separation efficiency, protein standards were utilized to test peak recoveries, band broadening, and throughput. The assembly showed the possibility of handling up to 50 μg of proteins without incurring overloading. The developed channel was applied to demonstrate size sorting of lipoproteins for the future study of size dependent lipidomic and proteomic analysis. The current trial offers a unique advantage of scaling up HF5 separation without using wide-bore, hollow fibers which sacrifice separation speed.

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1. Introduction

Hollow fiber flow field-flow fractionation (HF5 or HF FIFFF) [1–3] is utilized as an alternative FIFFF system by adopting a hollow fiber membrane as a separation channel, and its performance is comparable to typical flat channel systems having a rectangular cross-section. FIFFF is an elution-based separation method that has been employed for the size fractionation of water soluble polymers and biological materials like proteins, cells, bacteria, and subcellular materials, and for the simultaneous characterization of hydrodynamic diameters or diffusion coefficients [4–7]. Separation of particles or proteins in typical FIFFF channel systems is carried out in a thin rectangular channel via the application of a crossflow across the channel thickness, acting as an external field to retain sample components, with migration flow moving along the channel axis to drive sample components down the channel at different speeds according to the differences in hydrodynamic diameters. Therefore, a smaller molecule with a faster diffusion will extrude at a faster migration flow due to parabolic velocity profiles, and thus it elutes earlier than a larger one. Since FIFFF has the capability of fractionating biological materials by size without altering their

biological activities, it provides bio-compatibility for life science research.

Separation in an HF5 follows the simple FIFFF principle of size separation except that the radial flow, acting toward the inner wall of the fiber, replaces the role of the crossflow of a rectangular FIFFF channel due to the cylindrical geometry of a hollow fiber (HF). Due to the simplicity and the low cost of construction for HF module, its application increases in a number of areas. Since HF5 began with the separation of particles [1] or aqueous polymers [2] about 20 years ago, its performance has been found to be comparable to conventional FIFFF [3,8] and applied to synthetic polymers, proteins, protein drugs, bacteria and cells, and lipoproteins [9–14] along with instrumental advancements; minimization of HF module for microscale separation [15], pre-fractionation device for proteomics research; *Corynebacterium glutamicum* proteome [16], and blood serum [17], on-line hyphenation of HF5 with electrospray ionization-mass spectrometry (ESI-MS) for whole bacteria analysis [18] and with capillary isoelectric focusing (CIEF-HF5) for two-dimensional proteome fractionation devices [19]. Recently, HF5 was demonstrated to be efficient. Due to its disposability, HF5 also reduces the run-to-run carryover issue, which can be advantageous when dealing with biological or pathological samples. However, all of these works are based on analytical-scale separations; in cases where collection of some amount of sample fractions are required for secondary analysis, repeated injections of HF5 are required to accumulate the necessary sample fractions.

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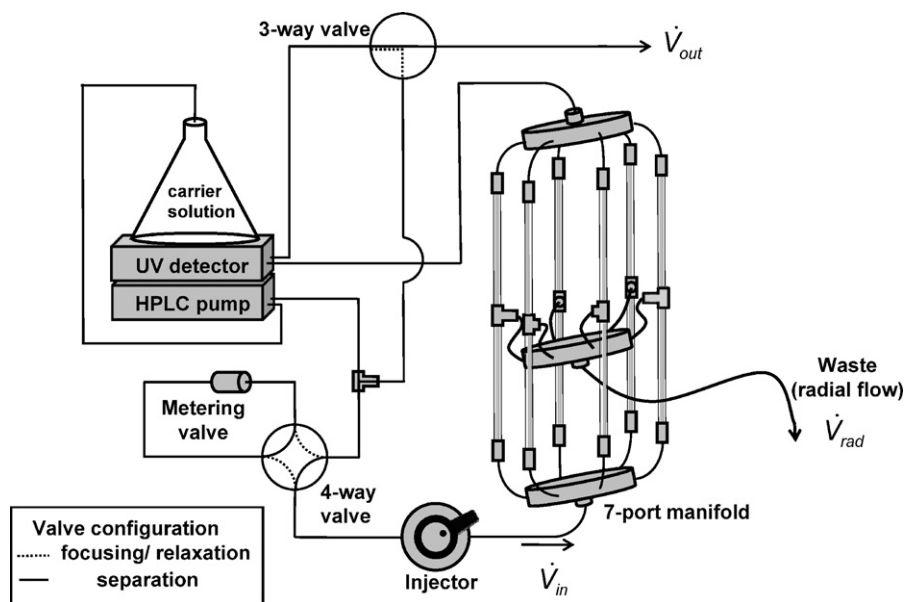


Fig. 1. Schematics of the multiplexed hollow fiber flow field-flow fractionation (MxHF5) system. Flow pathways of both valves are represented with dotted and solid line connections for focusing/relaxation and separation modes, respectively.

In this study, we introduce the multiplexed HF5 (MxHF5) system as a semi-preparative device applicable for biological separation. The concept of multiplexed FIFFF has been first made with circular asymmetrical FIFFF channel [20] which utilizes the similar flat type channel. The MxHF5 module in this study is constructed by a simple connection of six parallel HF5 modules, which provides an inexpensive construction and disposability to become an alternative for high throughput separation. This paper deals with the potential of MxHF5 for high throughput separation by evaluating the efficiency of the first MxHF5 in terms of band broadening, overloading, throughput, and peak recovery using protein standards. In addition, the developed system was applied for size sorting of lipoproteins from human blood plasma, a technique that can be utilized in the future for size specific proteomic and lipidomic analysis.

2. Experimental

The MxHF5 module was constructed in our laboratory by connecting six individual HF5 modules in parallel, as illustrated in Fig. 1. The HF material used in this study was polyacrylonitrile (PAN) from Chemicore Inc. (Daejeon, Korea) with dimensions of 1.0 mm \times 1.4 mm \times 25 cm (I.D. \times O.D. \times length) and a molecular weight cut-off of 30 kDa. Each individual HF5 module was constructed as described in our earlier reports [10,15]. A single hollow fiber was inserted into two pieces of Glass tubing (1.8 mm-I.D., 3.5 mm-O.D.) to act as module housings. The housings, as well as an HF, were then connected with a Teflon tee from Upchurch Scientific (Oak Harbor, WA, USA). The connection between the fiber module and Teflon connecting tubing (1.6 mm-O.D. and 0.254 mm-I.D.) leading to and from the fiber module used 1/8 in. hand-tight ferrules and nuts. The MxHF5 was assembled using two 7-port manifolds from Upchurch so that both six inlets and six outlets were connected to each manifold. Configuration of the MxHF5 system is illustrated in Fig. 1, which represents the flow path configurations during sample loading and focusing/relaxation mode with dotted lines and those of separation mode with solid lines.

The HPLC pump used in this work was a Model SP930D solvent delivery pump from Young-Lin Instrument (Seoul, Korea). The

pump flow was divided into two parts using a metering valve with a 1:9 flow rate ratio (represented with a dotted line on the left side of Fig. 1). One stream 1/10 of the total flow rate entered the channel inlet through an injector, a 7125, 90 μ L loop injector from Rheodyne (Cotati, CA, USA), and the other 9/10 of the total flow rate was directed into the channel outlet. During this process of focusing/relaxation, all flows exit through the fiber wall and induce radial flow. Sample components were introduced during this focusing/relaxation period through the injector and were expected to accumulate at a position 1/10 of the way down the fiber. The focusing/relaxation period was sustained for a sufficient time to assure sample components achieved equilibrium states between the diffusive forces protruding from the membrane wall and the hydrodynamic forces of radial flow acting toward the membrane wall. A 5 min focusing/relaxation period was used, in addition to the time required for sample materials to be delivered from the injector to the fiber. After focusing/relaxation, the three-way valve was switched to the separation mode so that the carrier flow was delivered to the channel inlet only while the radial flow rate was maintained. Eluted sample species were monitored at 280 nm by a model UV730D UV detector from Young-Lin Instrument (Seoul, Korea) and the detector signals were recorded using Autochro-2000 software from Young-Lin. For a precise balance of the radial flow and outflow rates, a needle valve was connected at the end of the detector outlet. The carrier solution used for MxHF5 was 10 mM NH_4HCO_3 solution prepared with ultrapure water ($>18 \text{ M}\Omega$) which was filtered before use with a 0.22- μ m membrane filter (Millipore Corp., Bedford, MA, USA). Protein standards were bovine serum albumin (BSA) (66 kDa), apoferritin (444 kDa), and thyroglobulin (670 kDa) from Sigma (St. Louis, MO, USA). The lipoprotein sample used in this study was human blood plasma from a healthy donor which was stained with Sudan Black B before injection into the MxHF5. Staining of lipoproteins in the plasma sample was achieved by mixing 100 μ L of raw plasma with 10 μ L of 1% Sudan Black B (SBB) in dimethylsulfoxide. The mixture was vortexed for 20 min and then stored overnight at 4 $^\circ\text{C}$. The mixture was injected directly into the MxHF5 with no further treatment. For the detection of lipoproteins during MxHF5 separation, a wavelength of 600 nm was selected.

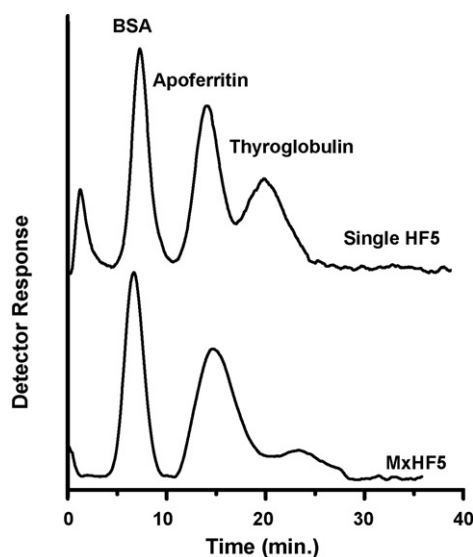


Fig. 2. Fractograms of protein separation by single HF5 and MxHF5 systems. Flow rate condition of the single HF5 was $\dot{V}_{out}/\dot{V}_{rad}$ (outflow rate/radial flow rate) = 0.12/0.38 mL/min. For MxHF5, flow rate was increased to six times ($\dot{V}_{out}/\dot{V}_{rad}$ = 0.72/2.28 mL/min) to that used for the single HF5.

3. Results and discussion

The MxHF5 module, a complex of six HF5 tubes, was evaluated for its separation efficiency of protein standards; the separation efficiency of MxHF5 was then compared with that of a single HF5 module. Fig. 2 is the comparison of the separations of three proteins (BSA, apoferritin, and thyroglobulin) using a single HF5 (top) and a MxHF5 (bottom). Experimental conditions for the MxHF5 run were the same as those for the single HF5, only scaled up for six tubes; the injection amount for each protein species in Fig. 2 was 2.5 μg each for the single HF5 and was increased to 15 μg each for the MxHF5 system. Flow rate conditions were $\dot{V}_{out}/\dot{V}_{rad}$ (outflow rate/radial flow rate) = 0.12/0.38 mL/min for the single HF5 and 0.72/2.28 mL/min for MxHF5. The protein separation shown at the top of Fig. 2 was accomplished within 25 min, with a slight overlap of the last two species. As seen in the lower case, thyroglobulin was

Table 1

Changes in retention time (t_r) and experimental plate height (H_{exp}) values for the three protein standards obtained with both the single HF5 and the MxHF5 module. The injection amount of each protein for the MxHF5 module was increased to six times that of the single HF5 module. Measurements were made with $n = 3$ for each set of data.

	t_r (min)		H_{exp} (cm)	
	Single HF5	MxHF5	Single HF5	MxHF5
BSA	8.0 \pm 0.1	8.0 \pm 0.1	0.15 \pm 0.02	0.20 \pm 0.02
Apoferritin	14.2 \pm 0.3	14.7 \pm 0.1	0.13 \pm 0.02	0.19 \pm 0.02
Thyroglobulin	20.9 \pm 0.1	22.0 \pm 0.1	0.09 \pm 0.01	0.14 \pm 0.01

not well resolved in the MxHF5. The retention time of each species does not appear to be significantly different between the modules. Retention times and experimental plate height values of the two channels are compared in Table 1. The retention time data for the three proteins in MxHF5 showed a slight increase (less than 5%) from those of the single channel as listed in Table 1, and band broadening occurred in MxHF5 to some degree. The plate height values of the MxHF5 increased by 33–56% compared to those of the HF5. A possible explanation for the band broadening of eluted components in MxHF5 is a slight variation of flow velocities among each individual fiber module since membrane pore uniformity of the hollow fibers may vary from different modules or from pressure changes in different tubing connections between 7-port manifold and each module. Another possibility may be the dead volume of the two 7-port manifolds located at both ends of the multiple HF5 modules. In order to test the peak recovery in the MxHF5 system, the peak area of BSA was measured for separate runs (3 μg for single channel and 18 μg for MxHF5). The MxHF5 module shows 71.0 \pm 3.6% ($n = 3$) while the single HF5 system shows 76.1 \pm 9.9%, and the latter value was similar to the value reported in the literature [10]. In this case, reference value for the 100% recovery was based on the measurement of peak area with the same amount of injection by fixing axial flow rate without applying radial flow. The maximum throughput of the MxHF5 module was investigated with the overloading effect by increasing the injection amounts of BSA, as shown in Fig. 3. As the injection amount was increased to 15 μg for the single HF5 and to 90 μg for the MxHF5, there appeared a shift in retention time to a shorter time region. From these experiments, it is found that about 50 μg of the protein sample can be

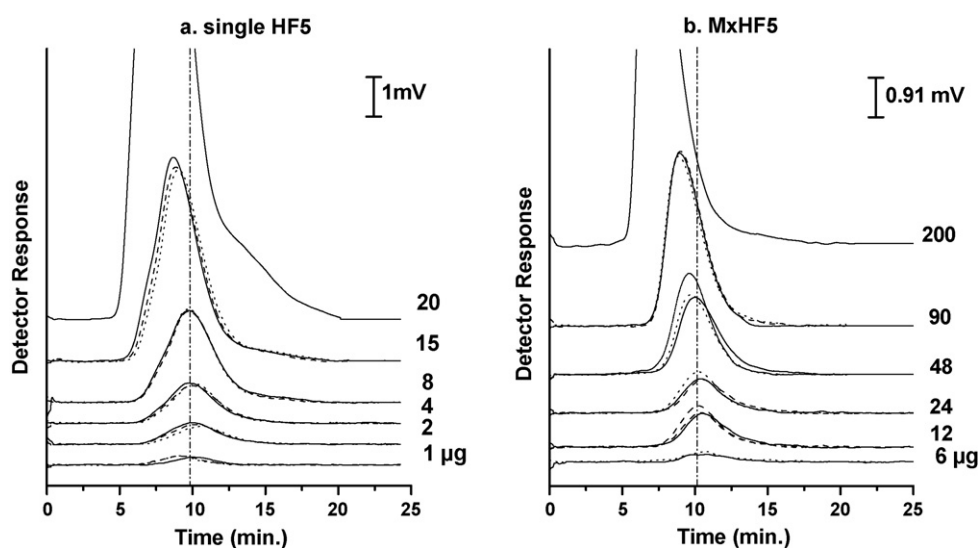


Fig. 3. Overloading of BSA observed for both single HF5 and MxHF5 systems by increasing the injection amount which are marked at the right of each fractogram. Flow rate conditions are $\dot{V}_{out}/\dot{V}_{rad}$ = 0.10/0.40 mL/min for the single HF5 (a) and 0.60/2.40 mL/min for the MxHF5 (b).

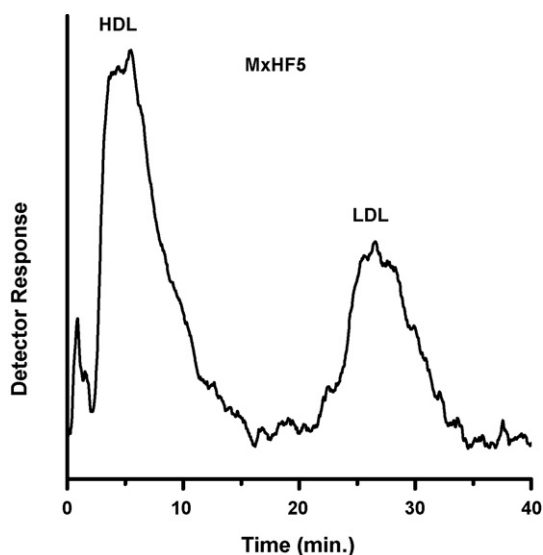


Fig. 4. Separation of lipoproteins from about 27 μ L of human blood plasma by MxHF5. Plasma sample was stained with Sudan black B before injection and lipoproteins were selectively detected at 600 nm. Flow rate condition was $V_{out}/V_{rad} = 0.80/3.00$ mL/min.

resolved with MxHF5 without overloading. The flow rate condition for the test illustrated in Fig. 3a was $V_{out}/V_{rad} = 0.10/0.40$ mL/min, and was scaled up for MxHF5, similar to the test in Fig. 2. The dashed or dotted lines in the fractograms represent the reproducibility of experiments.

The MxHF5 module was applied for the separation of lipoproteins contained in a blood plasma sample as shown in Fig. 4. Approximately 30 μ L of blood plasma mixture added to Sudan Black B prior to separation (equivalent to 27 μ L of pure blood plasma) was injected into the MxHF5 module for the separation of high density lipoprotein (HDL) and low density lipoprotein (LDL). The fractogram was recorded at a wavelength of 600 nm. We have demonstrated in an earlier study that the relative decrease in LDL sizes in a patient with coronary artery disease (CAD) can be monitored by FIFFF [21]. The current study shows the possibility of applying the MxHF5 for the semi-preparative sorting of lipoprotein particles, which can then be utilized for the secondary analysis of each lipoprotein particle type. This can be a useful means for systematic pattern analysis of phospholipids and other components contained in each lipoprotein type in relation to CAD by utilizing a systematic LC–ESI–MS–MS analysis often required for lipidomics and proteomics research. Subsequent analyses will be presented in future studies using off-line coupling of MxHF5 and nanoflow LC–ESI–MS–MS.

4. Conclusions

A simple multiplexed HF5 module for increasing throughput of the HF5 was developed by the parallel connection of six HF5 modules. The performance and efficiency were evaluated using protein standards. When the HF5 modules were hexaplexed, band broadening of eluting proteins was observed, with an increase of 56% of a single HF5 module, while changes in retention time and peak recovery were not significant. It was shown that up to ~ 50 μ g of protein molecules can be handled in the hexaplexed HF5 module without incurring an overloading effect. This trait can be utilized for the semi-preparative sorting of proteins or any biological particles in relation to size dependent proteomic or lipidomic analysis. In the case of lipoproteins from human blood plasma, the current work shows the possibility of collecting different sizes of lipoproteins in such a way as to carry out size specific analyses of biomarkers related to adult diseases such as CAD or obesity. The subsequent study to elucidate the differences in the patterns of phospholipids and proteins contained in different types of lipoproteins between healthy and unhealthy patient samples is in progress.

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