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Development of a Multilane Channel System for Nongel-Based Two-Dimensional Protein Separations Using Isoelectric Focusing and Asymmetrical Flow Field-Flow Fractionation

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A dual purpose multilane channel system to carry out isoelectric focusing (IEF) and asymmetrical flow field-flow fractionation (IEF-AFlFFF or IEF-AF4) was developed for the high-speed fractionation of a proteome in two dimensions (2D): isoelectric point (p*I*) and hydrodynamic diameter (d_s). Separation of proteins is initially achieved by differences in p*I* using IEF in an open thin segment, which is formed by interconnecting the beginning part of six parallel flow FFF channels in the lateral direction. After IEF, each protein pool of a different p*I* interval is simultaneously separated in an orthogonal direction by d_s in six individual AF4 channels. The developed IEF-AF4 multilane channel system provides ultimate nongel, elution based, and 2D protein separation at an improved separation speed; the entire separation can be processed within 30 min, compared to ~3 h with the previously developed capillary isoelectric focusing-hollow fiber FlFFF (CIEF-HFFlFFF or CIEF-HF5) (Kang, D.; Moon, M. H. *Anal. Chem.* 2006, *78,* 5789−5798) or ~36 h with 2D-polyacryamide gel electrophoresis (2D-PAGE). An initial evaluation of IEF-AF4 was performed to investigate the influence of ampholyte concentration and IEF voltage on the separation of standard protein mixtures.

Prefractionation of complex proteomes is one of the critical issues in proteomic studies because the complexity of proteomes exceeds the resolution capabilities of the current most sophisticated mass spectrometry (MS) techniques either by bottom-up or top-down approaches. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is widely being used as a primary technique for protein separation from complex mixtures due to its remarkable resolution.[1−3] However, it has some drawbacks, such as ineffectiveness in terms of operation speed and labor, difficulties in detecting low abundance proteins and in retrieving trapped proteins from gels without cleavage, and loss of conformational information of proteins due to denaturation. Since the separation power of multidimensional separation methods can be greatly enlarged by multiplication of the peak capacity of each individual method,[4] various nongel-based 2D separation schemes have been introduced in an attempt to address the limitations of 2D-PAGE while maintaining the capability to resolve complex protein mixtures. For example, size exclusion chromatography (SEC) was online coupled as a second dimension to capillary isoelectric focusing (CIEF) to separate some model proteins based on differences of hydrodynamic volumes and isoelectric points (p*I*s).[5] Online hyphenation of CIEF with other separation methods such as reversed phase liquid chromatography (CIEF-RPLC),[6−8] capillary zone electrophoresis (CIEF-CZE),[9] and capillary gel electrophoresis (CIEF-CGE)[10] has been tried for protein separation as well. An off-line three-dimensional (3D) separation method was introduced with strong anion exchange (SAX) chromatography for the first dimension, followed by RPLC of SAX fractions, and finally by 1D-PAGE of each RPLC fractions using fluorescence and isotope-coded protein labeling for quantitative proteomics.[11] However, these methods cannot avoid the possibility of sample loss during migration through packing media or gel networks, or denaturation of proteins due to the use of organic solvents or surfactants. Online combinations of CIEF with any CE method also require a separate microdialysis interface to remove the ampholyte solution needed for CIEF if MS analysis is to be continued. Recently, a microfluidic chip-based isoelectric focusing (IEF) with CE using monolith valves showed an ability to prefractionate proteins in a certain p*I* range.[12] Off-gel electrophoresis (OGE) for IEF was coupled to CE for 2D separation of peptides with a practical peak capacity of ~700.[13]

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As an alternative to these gel-based or packed bed separation systems, flow field-flow fractionation (F4) can be used to prefractionate complicated proteomes or cellular material due to the use of biofriendly buffer solutions in an obstruct-free channel. F4 is an elution method capable of separating all macromolecules or particulate materials (i.e., from proteins to whole cells ranging from 1 nm to 50 μm in size) by diffusion coefficients or hydrodynamic diameters,[14−16] and separation in F4 takes place in a thin, empty channel of rectangular cross-section using aqueous buffer solutions. However, separation in F4 is based on the use of an orthogonal field which drives sample components on one side of channel walls into different velocity regions within the parabolic flow profile of the migration flow moving along the channel axis. Since flow velocity in parabolic flow profiles increases away from the channel wall, sample components of different diffusion coefficients (or hydrodynamic sizes) migrate differentially, ultimately leading to a size-based separation. F4 has been coupled to MS either on-line or off-line for the separation of *E. coli*,[17] human hemoglobin,[18] the *Corynebacterium glutamicum* proteome[19] using a hollow fiber (HF) module (cylindrical F4 channel named HFFlFFF or HF5). Recently, the proteomic applicability of F4 was further demonstrated with size fractionation of the mitochondria of rat liver[20] and of exosomes from human neural stem cells[21] using miniaturized F4 systems.[22,23] Both were followed by nanoflow LC−ESI-MS/MS analysis for protein digests for size-dependent proteome profiling of mitochondria and exosomes, respectively.

In a recent effort to pursue 2D separation using F4, CIEF was online coupled to microscale HF5 (μHF5),[24] in which nongel-based 2D protein separation was effectively carried out by the sequential injection of p*I*-based fractions from CIEF (the first dimension) to the μHF5 to separate proteins based on differences in diffusion coefficients or Stokes' diameter, d_s, (the second dimension).[25] Similarly to 2D-PAGE, CIEF-μHF5 fractionates proteins based on differences in p*I*s and d_s, but it is a gel-free method which provides additional features such as isolation/collection of intact proteins in a certain p*I* and d_s interval due to the use of a biofriendly buffer in HF5 separation, online removal of carrier ampholine solution during μHF5 separation, and significant reduction of separation time (~3 h maximum). The earlier development has been applied to fractionate human urinary proteomes leading to the identification of 114 urinary proteins. However, in CIEF-μHF5, while one p*I* fraction of proteins is injected into μHF5 for size sorting, other p*I* fractions of lower or higher pH must remain in the CIEF tube until μHF5 separation of a previous fraction is completed. Even though CIEF-μHF5 reduced total operation time by about a factor of 10 from that of 2D-PAGE, stagnation of fractionated proteins in CIEF tubing under the electrical field caused unnecessary delay in the separation process and induced a shift in the fractionated sample band due to the electroosmotic flow (EOF). A relatively low throughput of CIEF-μHF5 compared to 2D-PAGE can be a minor point in some cases due to the use of a capillary for IEF and a microbore hollow fiber. To improve the separation speed and throughput of CIEF-μHF5 with the simultaneous minimization of the influence of EOF, a different geometrical scheme to the previous CIEF-μHF5 design needs to be introduced which maintains the excellent features of CIEF-μHF5 such as separation of intact proteins by 2D and online purification of proteins. Instead of the use of a capillary version of CIEF and HF5, the new multilane channel developed in this study adopts the rectangular design of an field-flow fractionation (FFF) channel with an array of multiple asymmetrical flow field-flow fractionation (AF4) channels.

In the new IEF-AF4 multilane channel system developed in this study, six AF4 channels are aligned in parallel as shown in Figure 1a and the beginning area of the six channels are connected to each other to make an IEF segment where IEF is carried out in a direction orthogonal to the channel axis. For IEF-AF4 separation, complex proteome samples mixed with ampholyte solution are injected into one of the two ports located at the IEF segment via a syringe pump while the opposite port is open for drainage. Once a proteome sample is loaded into the IEF segment, a voltage is applied from both ends of the segment for IEF for a very short period of time (~5 min) to minimize the influence of EOF. As soon as IEF separation is completed, separated p*I* fractions of proteins are translocated to AF4 channel segments for focusing/relaxation and followed by size separation of each p*I* fraction in six channels simultaneously without applying an electrical field. Focusing/relaxation[16] is a process to ensure equilibrium distribution of sample components in an AF4 channel by the balance of an external field (crossflow) and diffusion, which can be achieved in such a way that the two flow streams (one from the channel inlet and the other from the outlet) are focused to converge at a starting point of migration for a finite period of time. The starting point of migration in an IEF-AF4 channel system is adjusted at a position in the AF4 channel segment that is slightly apart from the IEF segment in Figure 1a. After focusing/relaxation, protein samples migrate along the channel axis by the application of flow only from the channel inlet and size fractionation of proteins can be made simultaneously at each channel. During AF4 separation, carrier ampholyte can be removed by crossflow movement of carrier solution (buffer solution) through the channel membrane layered below the channel spacer and, as a result, collected proteins preserve their conformations without being denatured. This can be advantageous in avoiding the protein purification work to remove the ampholyte solution when consecutive MS analysis of collected proteins is required and in differentiating intact proteins with post-translational modification.

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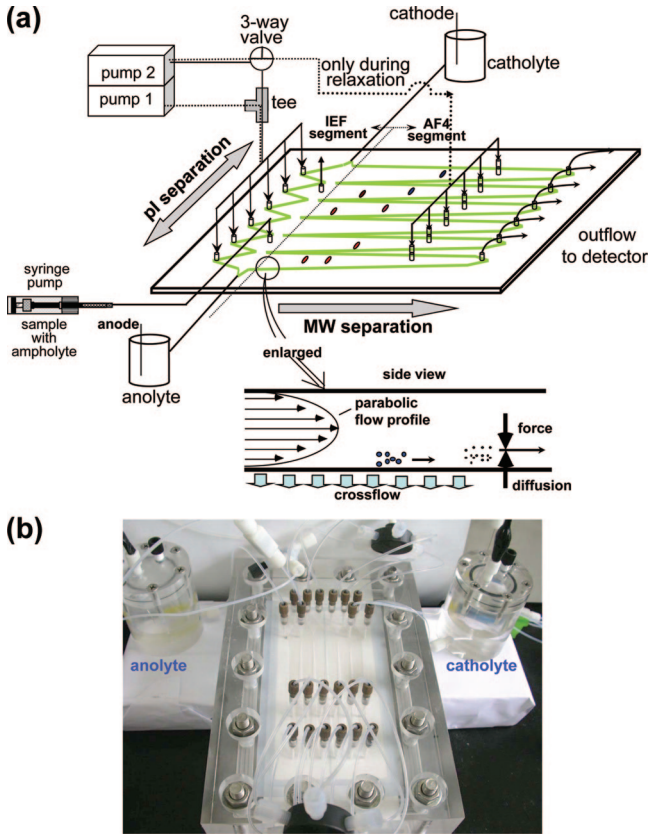

Figure 1. (a) Schematics of multilane FIFFF channel structure for IEF-AF4. A mixture of proteins and ampholyte solution is loaded from a syringe pump in a lateral direction into six parallel channels in the open IEF segment. After IEF, protein bands are transferred to each channel lane for MW separation by asymmetrical flow field-flow fractionation. (b) Photograph of a prototype multilane IEF-AF4 channel.

In this experiment, a prototype multilane channel system for IEF-AF4 was evaluated with protein standards by examining the influence of applied voltage and concentration of carrier ampholyte on IEF-AF4 using an ampholyte with p*I* values from 3 to 10. In experiments that increased both the throughput and speed of separation, the effect of electrolyte stacking on IEF is explained with reference to recovery and reproducibility. With this new strategy, high speed 2D (p*I* and d_s) intact protein separation can be achieved, and the entire operation could be fully automated with improved speed (less than 20−30 min) and throughput.

EXPERIMENTAL SECTION

Materials and Reagents. The protein standards used for the evaluation of IEF-AF4 separation were cytochrome C (12.4, p*I* 6.8), carbonic anhydrase (CA, 29 kDa, p*I* 5.8), bovine serum albumin (BSA, 66 kDa, p*I* 4.8), alcohol dehydrogenase (ADH, 150 kDa, p*I* 6.2), and apoferritin (444 kDa, p*I* 5.4) from Sigma (St. Louis, MO). Ammonium bicarbonate used for the preparation of the carrier solution of AF4, phosphoric acid for the anolyte solution, sodium hydroxide for the catholyte solution, and the ampholyte solution (Fluka Ampholyte High-Resolution pH 3 −10) were also purchased from Sigma. All aqueous solutions were prepared with ultrapure water (>18 MΩ cm) and filtered through a nitrocellulose membrane filter (0.22 μm) (Milipore, Danvers, MA).

Construction of the IEF-AF4 Multilane Channel System. The prototype IEF-AF4 multilane channel was built in-house in a manner similar to the construction of the miniaturized AF4 channels reported in our laboratory.[22,23] The multilane channel shown in Figure 1a consists of six parallel, trapezoidal channels between which spaces were made by cutting a 300 μm thick Teflon spacer. The tip-to-tip length, L_{tt}, of each channel is 11.0 cm, with an initial breadth of 1.0 cm that decreases to a final breadth of 0.3 cm. The lengths of the triangles at both the inlets and outlets are 1.0 and 0.3 cm, respectively. The beginning part of all six channels (between 1.0 to 1.5 cm from each channel inlet) is connected to make a segment for IEF as shown in Figure 1a. In this IEF segment, protein mixtures with ampholyte are loaded from the inlet via a KDS100 syringe pump from KD Scientific (Holliston, MA) (shown in Figure 1a). The length of the AF4 channel segment is 9.5 cm from the beginning of the channel divider to the end of each channel. Each effective channel area for AF4 separation right next to the IEF segment (between the beginning point of each individual AF4 channel to the end of each channel outlet) is 6.03 cm^2 and the geometrical channel volume of each AF4 channel is 0.18 cm^3. The channel spacer shown in Figure 1 a and a sheet of membrane, PLCGC (MWCO, 10 kDa) from Millipore Corp. (Danvers, MA), are sandwiched by two plastic blocks as shown in Figure 1b (only the upper block image appears). The membrane is layered above the accumulation wall, the bottom block of the assembly shown in Figure 1b, and a water-permeable ceramic frit with 5 μm pores is embedded at the accumulation wall to allow for crossflow to pass through. The other wall (called the depletion wall), the upper block of Figure 1b, is made with a plain plastic block without a frit and thus the channel spaces are visible. Tubing connections to and from the IEF-AF4 channel are made with Teflon tubes with a diameter of 0.0254 cm with Sealtight PEEK fittings from Upchurch Scientific (Oak Harbor, WA). Anolyte and catholyte solutions are in contact with the IEF segment by using each separate reservoir through Teflon tubings. Electrolyte reservoirs are made of plastic blocks with cylindrical chambers (20 cm^3). Each reservoir is filled with 0.015 M phosphoric acid for the anolyte solution and 0.015 M NaOH for the catholyte solution. When sample mixtures are loaded onto the IEF segment by a syringe pump, all inlets and outlets of the IEF-AF4 channel and the crossflow outlet are closed, except for the outlet of the IEF segment so that proteins with ampholyte mixtures are placed between the two ports. After sample loading, an electrical field is supplied via Pt wires immersed in both reservoirs with a model 205B-10R high-voltage power supply from Bertan (Hicksville, NY) for IEF. The electric voltage range used is 1∼3 kV for 5 min, which was optimized after visual observation of forming a sample band using colored protein standards.

The AF4 separation is performed using two identical model 590 HPLC pumps (Waters, Milford). The carrier solution for AF4 separation is a 10 mM NH_4HCO_3 solution prepared from ultrapure water (>18 MΩ cm). Operation of AF4 is divided into two steps: focusing/relaxation and migration for fractionation. Right after IEF operation, protein bands fractionated by p*I* differences in the IEF segment are immediately transferred to the beginning of each AF4 channel, and then each protein fraction undergoes the focusing/relaxation procedure to es-

tablish equilibrium states of proteins before separation. This is achieved by delivering carrier solution from both ends of each channel with adjustments of the flowrate ratio. Flow splitting to each of the six inlets is made by connecting a PEEK seven-port manifold from Upchurch Scientific. The time period for allowing focusing/relaxation to occur is 100 s, which is sufficient for both the relocation of protein bands from the IEF segment to the beginning end of each AF4 channel segment, and relaxation. After focusing/relaxation, pump 2 is stopped and only the flow stream from pump 1 is delivered to the inlets of the AF4 channel at an increased rate for AF4 separation. During AF4 separation, proteins in each AF4 channel are fractionated by an increasing order of hydrodynamic diameter and the eluting species are monitored by using model UV M720 absorbance detectors from Young-Lin (Seoul, Korea) at 280 nm and the signals recorded using AutochroWin software provided by Young-Lin.

RESULTS AND DISCUSSION

Performance of the IEF-AF4 multilane channel was evaluated with protein standards. Prior to the proteins with ampholyte solution being loaded into the IEF segment of the multilane channel, the interface between each electrolyte reservoir and the IEF segment needed to be filled with electrolyte solutions. Because ampholyte solution was pulled out of the IEF segment and toward each electrode, a loss of proteins having extreme p*I*s close to the limiting end of the p*I* interval of the ampholyte, such as cytochrome C (p*I* 10.3) or abumin (p*I* 4.8), was observed when electrolyte solution was not filled in the interface (triangular space at both ends of IEF segment in Figure 1) as mentioned above. This will be explained later in detail. To fill the connection interface with electrolyte, ampholyte and catholyte solutions were pushed from the reservoir to the IEF segment by opening the sample inlet and outlet as shown in step-1 of Figure 2a. Then, the IEF segment was washed (step-2) with water as shown with a solid line configuration of pump 2 in Figure 1a. After the channel was cleaned with water, ampholyte solution with protein mixtures was loaded onto the IEF segment of the multilane channel using a syringe pump (step-3). The total injection volume to the IEF segment was 20 μL. For IEF, an electrical field was applied for 5 min while all the inlets and outlets of the multilane channel were blocked. Right after IEF, fractionated protein bands were transferred to the beginning end of each AF4 channel segment by pumping carrier solution (10 mM NH_4HCO_3) for AF4 separation. This was achieved by focusing two counter-directing flow streams; one from pump 1 and the other from pump 2 as shown by the dotted line in Figure 1a. Focusing flow streams delivered through the six ports located in the middle of AF4 channels (8.0 cm from the channel inlets and shown with a dotted line in Figure 1a) by pump 2 were used only when focusing/relaxation was needed. At the same time, carrier flow from pump 1 was delivered to the channel (to six channel inlets equivalently) so that the two opposite flow streams could be focused at a location right next to the beginning end of each AF4 segment. This was controlled by adjusting the ratio of the two flow rates (approximately 1:3 by visual optimization using dye). This is similar to a typical focusing/relaxation procedure commonly required in the operation of an AF4 system. At the same time, the outflow stream of each channel leading to each detector was also controlled by applying back-

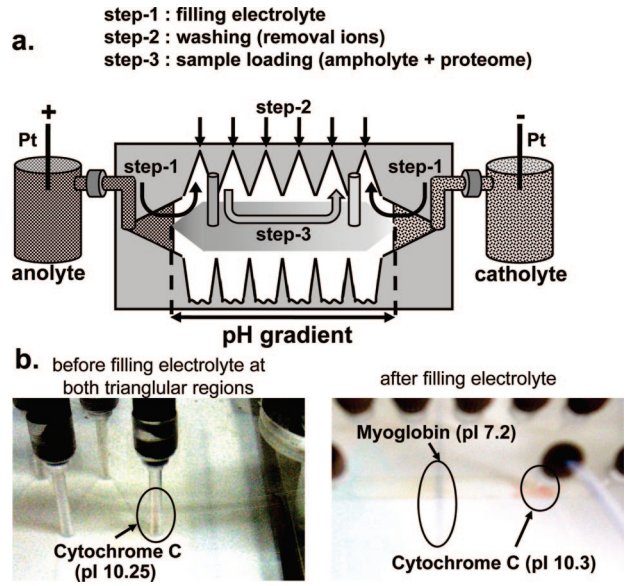

Figure 2. (a) Filling of both ends of the tubing connection to the IEF segment with electrolytes to keep protein samples from exiting the channel toward each electrode at step-1, washing with water at step-2 to remove ions, and sample loading at step-3. (b) Photographs of cytochrome C (shown with red color) at the end of the IEF segment (toward the cathode side) before and after adding electrolyte.

pressure with the length control of a capillary (i.d. = 100 μm) at the end of detector so that only the desired flow rate could exit; the rest of the flow in the multilane channel should exit through the accumulation wall as crossflow. The reason to let the detector be flushed with carrier flow is to maintain the detector baseline steady without incurring an abrupt change. Focusing/relaxation was carried out for a period of 100 s, which was sufficient both for relocating the sample band to the AF4 segment and for sweeping one channel volume by crossflow for relaxation of sample components. This period is also dependent on the crossflow rate. After focusing/relaxation, pump 2 was stopped and the flow stream from pump 1 was directed only to the inlet of the channel for AF4 separation at an increased rate for which the total influx should be the same as that during focusing/relaxation.

During separation in the AF4 segment, proteins of different p*I* intervals are expected to be separated by hydrodynamic diameter in each channel. Ideally, the p*I* interval of proteins entering each AF4 channel is expected to be ΔpH = 1.16 (for six lanes in the case of ampholyte having pH 3−10), so that channel 1 (closest to the anode) corresponds to a pH interval of 3.00−4.16. However, when the interface between the reservoir and the IEF segment was not filled with electrolyte solution, cytochrome C (p*I* 10.25) did not appear in channel 6 and BSA (p*I* 4.8) eluted at the first AF4 channel (data not shown). This shows that the IEF of protein standards was not appropriately made and proteins were further attracted toward both electrodes. To test this hypothesis, 30 μg of cytochrome C was injected for visual examination, and photographs of the cathodic end of the IEF segment (channel 6) before and after filling interfaces with electrolytes are shown in Figure 2b. Without the addition of anolyte and catholyte to both triangular interfaces between the IEF segment and Teflon tubing, cytochrome C appeared red in the connecting tube leading to the cathode, which showed that it was pulled out of the IEF segment.

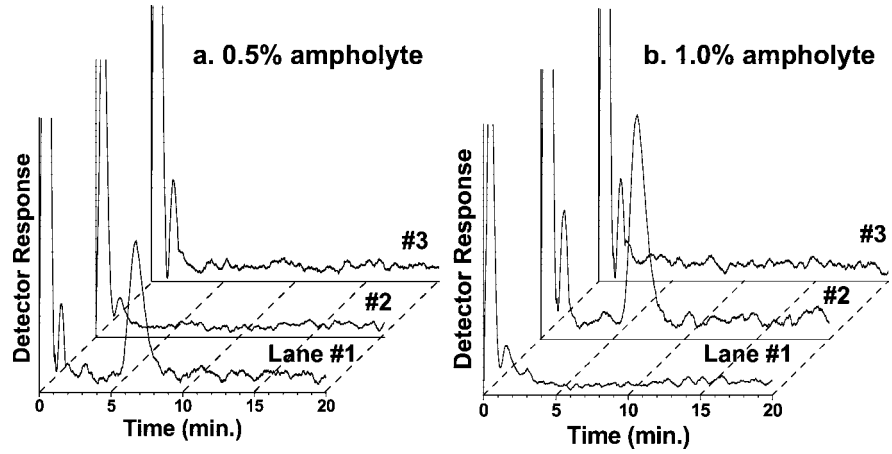

Figure 3. Effect of ampholyte concentration (0.5 and 1.0%) on IEF-AF4 separation of BSA (p*I* = 4.8, 66 kDa, injection amount = 1.0 μg). Ideal pH range of each FFF channel is 3.00−4.16, 4.17−5.33, and 5.34−6.50 for lanes 1, 2, and 3, respectively. The applied electric voltage is 3 kV for 5 min, and the flow rates for AF4 separation at each channel lane are $\dot{V}_{in}/\dot{V}_{out}$ = 1.0/0.25 in mL/min (focusing/relaxation period for AF4 = 100 s).

However, after adding electrolytes, cytochrome C appeared to have been left inside the IEF segment right next to the channel lane 6 and it eluted in the channel lane 6 (the fractogram is not included here). To keep proteins from exiting the IEF segment, the IEF focusing period and applied voltages were tuned. The possibility of the electroosmotic flow (EOF) effect cannot be excluded when cytochrome C exited the IEF segment. However IEF was carried out only for 5 min under 3 kV which was not a strong electrical field condition to incur a strong EOF at polymeric channel surfaces (regenerated cellulose and acrylic block). A similar observation in the opposite direction was made with BSA (p*I* = 4.8) which was supposed to elute at the AF4 channel lane 2 (ideal pH range = 4.17−5.33 based on the assumption of the linear pH gradient), but it eluted at the channel lane 1 (ideal pH range = 3.00−4.16) without filling the electrolyte at the anodic side of the IEF segment. This supports that the pH gradient was formed over the entire IEF segment including both triangular ends and proteins were attracted toward each electrode due to the incomplete filling of the electrolyte at the triangular ends of the IEF segment which leads to ampholyte solution expanded toward the ends of the IEF segment. In order to allow a pH gradient established within the boundary of the IEF segment without triangular ends, the electrolyte filling process shown in Figure 2a was applied for all the following experiments prior to IEF.

Figure 3 shows the effect of ampholyte concentration on IEF of 1.0 μg of BSA, represented by AF4 fractograms that were observed from the three nearby channel lanes (numbers 1, 2, and 3). An electrical voltage was applied at 3 kV for 5 min for IEF. After IEF, the electrical field was turned off and the focusing/relaxation for sample components was begun. The accumulation of sample components by focusing/relaxation was placed at a position about 5 mm from the channel divider of the AF4 channel segment by applying two counter-directing flow streams at 1.0 mL/min (total flux at each channel lane). The crossflow rate was maintained at 0.75 mL/min for 100 s. These were the effective flow rates at each individual AF4 channel. After the focusing/relaxation procedure, AF4 separation of BSA began at flow rate conditions of $\dot{V}_{in}/\dot{V}_{out}$ = 1.0/0.25 in mL/min for each channel lane. Since the p*I* of BSA is known to be 4.8, BSA was expected to elute from channel 2 (ideal pH = 4.17−5.33 based on the assumption of a linear pH gradient). When the ampholyte concentration was 0.5%, BSA was found to elute from channel 1 which was beyond the expected pH interval (Figure 3a). This indicated that the pH gradient was not properly built up in the IEF segment due to low concentrations of ampholyte. However, when the ampholyte concentration was increased to 1.0%, BSA eluted exclusively from channel 2 of which the ΔpH includes the p*I* of BSA. A further increase in ampholyte concentration did not improve IEF resolution but increased the operation time required for IEF. The tall peak shown at the beginning of each fractogram is the void peak that is often observed in an AF4 system due to pressure change caused by the conversion of the flow direction after focusing/relaxation or some unretained species in the sample solution. In our experiments, a split void peak (a small tailing peak right after the main void peak) was observed throughout the experiment as shown in Figure 3.

Electrical field strength also influenced IEF resolution during IEF-AF4 operation. Tests were made with carbonic anhydrase (CA, p*I* 5.8) mixed with 1.0% ampholyte concentration. Figure 4 shows the AF4 fractograms of 1.0 μg of CA detected at three nearby AF4 channels (numbers 2, 3, and 4) at two different voltages, 1.0 and 3.0 kV. These voltages were applied over 6.5 cm length of IEF segment including each triangular interface. Flow rate conditions were the same as those used in Figure 3. At 1.0 kV, IEF of CA was not completed during a time interval of 100 s because CA molecules eluted from all three channel lanes. However, when the voltage was increased to 3.0 kV, CA appeared to elute exclusively from channel 3 (ideal pH = 5.34∼6.50) with a sharp peak.

On the basis of the examinations of the effects of ampholyte concentration and electrical field on IEF separation described above, a mixture of four protein standards (5 μg each of CA, BSA, alcohol dehydrogenase, and apoferritin) was injected for IEF-AF4 separation under 1.0% ampholyte concentration and 3.0 kV. Figure 5 shows a comparison of protein separation in an AF4 channel before and after IEF. The fractogram at the top of Figure 5 shows the separation of four protein mixtures using an AF4 channel without carrying out IEF. Flow rate conditions are the same as

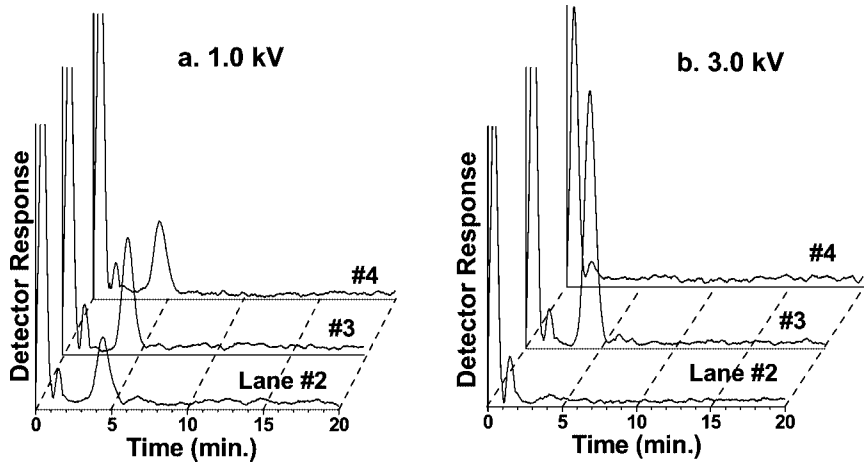

Figure 4. Effect of IEF voltage (1.0 and 3.0 kV) on IEF-AF4 separation of carbonic anhydrase (p*I* = 5.8, 29 kDa, injection amount = 1.0 μg). Ideal pH range of each FFF channel is the same as reported in Figure 3. The concentration of ampholyte is fixed at 1.0%. Run conditions for AF4 separation in each channel lane are the same as those used in Figure 3.

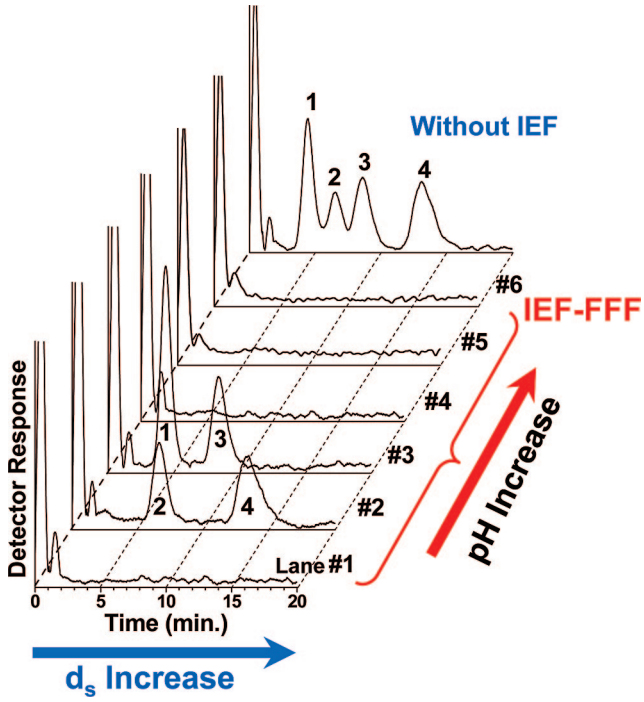

Figure 5. The AF4 fractograms of protein mixtures without and with IEF: 1, carbonic anhydrase (29 kDa, p*I* 5.8); 2, BSA (66 kDa, p*I* 4.8); 3, ADH (alcohol dehydrogenase, 150 kDa, p*I* 6.2); 4, apoferritin (444 kDa, p*I* = 5.4). Flow rates are the same as used in Figure 4. The ideal pH interval of each channel lane is expected to be nearly 1.16 (i.e., pH = 3.00−4.16 for lane no. 1).

those used in Figure 4. While the four mixtures were not completely separated by baseline resolution, it shows a relatively fast separation and also demonstrates the protein separation capability of the current AF4 channel used in this study. In this case, the same sample mixture (containing ampholyte) was injected into the IEF segment and transferred directly to AF4 separation without IEF. The fractogram at the top of Figure 5 shows no evidence that the ampholyte solution was eluted and monitored by the detector. Because the huge void peak at the beginning of separation in Figure 5 was found to be a pressure pulse after a blank injection of water, it was expected that the ampholyte would be filtered out during AF4 separation through the channel membrane. The top fractogram showing the separation of four protein components was obtained at channel 3. After IEF was carried out before AF4 separation, AF4 separation in each individual channel showed that protein numbers 2 (BSA, p*I* = 4.8) and 4 (apoferritin, 444 kDa, p*I* = 5.4) eluted in channel 2 (ideal pH = 4.17−5.33) and proteins 1 (CA, 29 kDa, p*I* = 5.8) and 3 (ADH, 150 kDa p*I* = 6.2) eluted in channel 3 (ideal pH = 5.34∼6.50). By comparison of p*I* values of proteins with the p*I* interval of each channel lane, all proteins eluted at their corresponding channels except for apoferritin (p*I* 5.4) which eluted from channel 2 (ideal pH interval: 4.17−5.33). This supports that the pH interval of each channel may not be linearly formed in the IEF segment. It needs to be further examined for the pH gradient in the IEF segment, but this is not covered in this study. However, these results demonstrate that the multilane channel system for IEF-AF4 provides 2D separation (by p*I* and MW) of proteins at less than 30 min, including 10 min of IEF, which is a significant improvement compared to ∼3 h for the previous CIEF-HF5. Recovery values during protein separation in IEF-AF4 were examined by measuring the peak area of BSA in AF4 channels with or without IEF. When IEF was not performed, peak recovery of BSA (5.5 μg of each injection) was calculated by measuring the peak area of BSA eluted at all lanes, which was found to be 72.3 ± 3.6% (n = 3) and is similar to recovery values reported for a miniaturized AF4 system in earlier work.[23] However, with IEF, the peak recovery decreased to 60.9 ± 4.9%, indicating that only about 85% of proteins are recovered by the IEF process. Reproducibility in repeated runs of BSA by IEF-AF4 yielded less than 4% RSD in peak area and 1.3% RSD in retention time measurements. Initial evaluation of the prototype IEF-AF4 system exhibited good reproducibility, but recovery values were relatively low. One possible way to improve relative recovery is to introduce a frit inlet type of the AF4 channel, FI-AFlFFF,[22] which gives a relatively higher peak recovery (larger than 80%) among F4 systems due to a nonstop separation process without a focusing/relaxation procedure.

CONCLUSIONS

The IEF-AF4 multilane channel developed in this study is capable of offering a nongel-based 2D (p*I* and MW) protein separation in the liquid phase. In contrast to the previous CIEF-μHF5,[25] which was constructed with a Teflon capillary and a microbore hollow fiber FlFFF, the present channel preserves most features of a CIEF-μHF5 system; nongel-based 2D separation of proteins, retrieval of intact proteins without losing their conformation, and online removal of ampholyte solution during the FFF separation stage which is useful when MS analysis is to be performed via on-line or off-line. However, the current multilane channel offers even faster separation than CIEF-HF5. Because HF5 separation of pH fractions in CIEF-μHF5 is not performed simultaneously, the operation time becomes longer as the number of pH fractions required for separation increases. In contrast, in the IEF-AF4 multilane system, the second dimension of separation (size separation by AF4) is carried out in multiple channels simultaneously. The entire operation takes less than 30 min (less than 10 min for sample loading and IEF and 20 min for AF4 separation). Compared to 2D-PAGE, which normally takes more than 36 h, the entire operation time of the current multilane channel system is significantly reduced. Automation is another advantage of the current system too. Moreover, the present channel was built on a large scale by adopting typical rectangular channel design in FFF and thus sample throughput can be increased compared to the limited injection amount (maximum $\sim$40 μg for the case of urinary proteome) required for CIEF-HF5. The current multilane channel is expected to accommodate more than a 100 μg of proteome sample since each channel lane handles more than 20 μg of protein standard, which can be increased further when channel dimensions (thickness and breadth) of each AF4 are enlarged.

A possible application of the IEF-AF4 channel is to fractionate proteins of specific p*I* and MW interval in intact states, when target proteins or biomarkers are known and their relative regulation is being examined. It can also be useful for the separation of low-abundance proteins (LAPs) from high-abundance proteins (HAPs), which often hinders successful identification of LAPs in shotgun proteomic analyses. Further studies are needed to evaluate the maximum throughput of this channel system for real proteome samples. If mechanically possible, AF4 channels with more numbers as needed should be incorporated in parallel so that smaller interval pH fractions can be processed with MW separation simultaneously.

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