Size Characterization of Sodium Hyaluronate by Field Programming Frit Inlet Asymmetrical Flow Field-Flow Fractionation/Multiangle Light Scattering

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Sodium hyaluronate (NaHA), water soluble polymer having ultra-high molecular weight, is characterized by using on-line frit inlet asymmetrical flow field-flow fractionation (FI-AFlFFF) and multiangle light scattering (MALS). This study demonstrates the capability of power programming FI-AFlFFF for the separation of NaHA and the applicability of FI-AFlFFF with MALS for the characterization of molecular weight distribution and their structural information. Since sample injection and relaxation in FI-AFlFFF are achieved by using hydrodynamic relaxation, separation of high molecular weight polymers can be achieved smoothly without halting the separation flow. Experiments are carried out with the two different NaHA products (a raw NaHA sample and a thermally degraded NaHA product) and molecular weight distribution and conformations in solution are determined. Influence of sample filtration on the change of molecular weight distribution is also discussed.

Key Words : Field-flow fractionation, Frit inlet asymmetrical flow field-flow fractionation, Molecular weight determination, Multiangle light scattering, Field programming

Introduction

Sodium hyaluronate (NaHA), a sodium salt of hyaluronic acid, is a natural and very high molecular weight linear polysaccharide composed of disaccharide repeating unit (D-glucuronic acid and N-acetyl-D-glucosamine). NaHA has been found in various body tissues and fluids such as vitreous humour, umbilical cord, and etc. and it is involved with many biological functions including lubrication of joints, regulation of molecular permeation into tissues, wound healing, inflammation, and etc.[1-4] Pharmaceutically, NaHA has been utilized for the hydrogel formation, drug attachment, protection of cells and tissue, and as a substitute for vitreous humor after opthalmic surgery.[1,2] The molecular weight of NaHA found has been known as few millions in Daltons and the size characterization of NaHA in aqueous solution is important for the desired application. Determination of molecular weight of NaHA has been mostly carried out by size exclusion chromatography (SEC),[1,5] capillary electrophoresis,[6] and ion exchange chromatography.[7] Approaches to measure molecular weight of NaHA with SEC were carried out with viscometric measurements,[4,8,9] matrix assisted laser diffraction ionization mass spectrometry (MALDI-MS),[2] and low-angle laser light scattering (LALLS).[10] In the case of using light scattering technique, it is accurate only for samples of narrow molecular weight distribution. Otherwise, sample components of a broad MW distribution must be fractionated by some means before measurements. In case of using SEC, a traditional size separation technique for polymers, difficulties to obtain an accurate molecular weight distribution arise from a lack of suitable calibration standards for NaHA, a loss of resolution for samples larger than the size exclusion limit of SEC column, and a possible degradation of polymeric chain due to the shear when penetrating pores of packing materials or a possible sample adsorption at the surface of packing materials. Recently, flow field-flow fractionation (FlFFF) with an on-line combination of multiangle light scattering (MALS) detection was utilized for the separation and simultaneous characterization of NaHA.[11,12]

FlFFF is an elution based separation technique that is a useful alternative to SEC for the separation and size characterization of aqueous polymers.[13] Since FlFFF separation is carried out in a thin, empty, ribbon like channel space, it is well suited to fractionate large macromolecules and particulate materials.[14-17] When sample materials are placed to an FlFFF channel, they are pushed toward the one side of channel wall (accumulation wall) by the applied field (cross flow). During a short period of time, sample components find equilibrium positions where the field force and the diffusion are counterbalanced each other, and then sample components are differentially distributed against the accumulation wall according to their sizes. When an axial flow is applied, they will migrate at different speeds due to the parabolic flow profiles in a thin channel. Therefore, polymers of a smaller MW will elute earlier than those of a larger one. Since the on-line coupling of MALS to FlFFF has been attempted for the size characterization of polystyrene latex and a dextran in 1994,[18] the joint technique has become a successful method to separate and characterize molecular weight and their distributions of water soluble polymers, such as polysaccharides, cellulose, polyacrylamides, and etc.[19-27] A number of FlFFF/MALS applications have

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been carried out mostly with asymmetrical FlFFF (AFlFFF) which has only one permeable wall at the accumulation wall.[18-22] In AFlFFF, sample migration is temporarily halted for achieving equilibrium states after sample injection, and then the separation begins. This is called as focusing/relaxation.[15] While AFlFFF utilizes a focusing/relaxation procedure which requires stopping sample migration for a period of time and may bring a possible sample adhesion at the channel wall, it has been widely used as an efficient and rapid separation method for high molecular weight water soluble polymers. A first study of FlFFF/MALS for the size characterization of NaHA was also carried out with asymmetrical FlFFF channel.[11]

Recently, frit inlet asymmetrical FlFFFF (or FI-AFlFFF),[17,28,29] a modified form of FlFFF channel suitable for the stopless separation, was utilized with MALS for the separation of NaHA[12] and the effects of field programming parameter and ionic strength of carrier solution on the separation of NaHA were evaluated. FI-AFlFFF was developed to bypass the focusing/relaxation procedure.[17] During sample injection in FI-AFlFFF, sample components are pushed toward the accumulation wall by the high speed frit flow as shown in Figure 1 and they are transported to their equilibrium positions continuously without stopping the migration. Thus, sample relaxation can be achieved hydrodynamically and the separation begins continuously. Therefore, it is advantageous in reducing the fear of sample adhesion at the accumulation wall and the entire system operation is simplified. In addition, field programming can be employed very easily to an FI-AFlFFF channel system by circulating the cross flow to the frit flow and reducing the circulation flow rate with time.[28] Compared to other FFF techniques (sedimentation FFF or thermal FFF), field programming technique has been rarely used in FlFFF except few cases.[16,19,25,28] In a previous study,[12] field programming FI-AFlFFF was coupled with MALS for the separation of a broad molecular weight NaHA material and the study was focused to evaluate field programming parameter for the successful separation of NaHA along with an investigation of ionic influence of carrier solution on separation. In this study, programmed separation of NaHA by FI-AFlFFF/MALS is applied for the determination of the difference in molecular weight distribution of a raw NaHA and a thermally degraded NaHA product, of which the latter has a different application in pharmaceutical or medical purposes depending on the molecular weight range. The difference in conformations between the two NaHA samples is discussed by examining the molecular weight dependence of radius of gyration for both samples. The change of size distributions of NaHA samples by sample filtration is also explained.

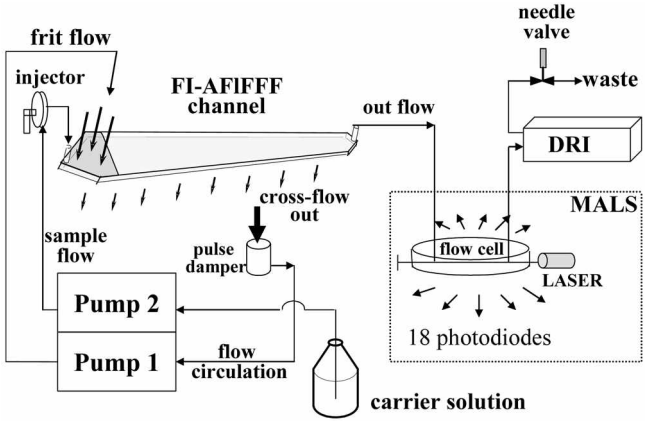

Figure 1. System configuration of the on-line frit-inlet asymmetrical flow field-flow fractionation/multiangle light scattering/differential refractive index detection (FI-AFlFFF/MALS/DRI). Field programming was achieved by circulating the cross-flow to frit flow.

Theory

Multiangle Light Scattering. Measurement of scattered light is well known to provide an absolute molecular weight according to the following theory as[30]

$$\frac{Kc}{R_\theta} = \frac{1}{M_w} + 2A_2c \tag{1}$$

where R_θ is the excess Rayleigh factor which is scattering intensity excess to solvent scattering for solute concentration c, M_w is the molecular weight, A_2 is the second virial coefficient, and K is a scattering constant given by

$$K = \frac{4\pi^2 n_0^2 (dn/dc)^2}{\lambda_0^4 N_A} \tag{2}$$

in which λ_0 is the wavelength of an incident light, n_0 the refractive index of the solvent, and dn/dc the refractive index increment with concentration, and N_A the Avogadro's number. For the case of large particles or polymers of a very large molecular weight, where the radial axis of particles is larger than about 5% of the wavelength, a special form factor $P(\theta)$ is required to compensate the phase difference caused by the scattering of light at different parts of the particles or large macromolecules as

$$P(\theta) = 1 + \frac{16\pi^2 \langle r_G^2 \rangle}{3\lambda_0^2} \sin^2\left(\frac{\theta}{2}\right) \tag{3}$$

where $\langle r_G^2 \rangle^{1/2}$ is the root-mean-square radius (RMS radius) or radius of gyration. Thus, combined equation yields as[30]

$$\frac{Kc}{R_\theta} = \frac{1}{P(\theta)}\left[\frac{1}{M_w} + 2A_2c\right] \tag{4}$$

The above equation can be used to calculate absolute mass and radius of gyration by measuring scattered light intensities at different angles with MALS for each volume slice of NaHA that is eluted during FI-AFlFFF run.

Experimental Section

Reagents. The carrier solution used for the separation of NaHA was prepared with deionized water (>18 MΩ/cm)

containing 0.1 M $NaNO_3$ with 0.02% (w/v) NaN_3 added for bactericide. In all cases, the carrier solution was filtered through a membrane filter having pore size of 0.02 μm. Both samples (a raw and a modified NaHA) were obtained from LGCI (Daejeon, Korea). The raw NaHA material was extracted from Streptococus and the modified product was thermally degraded by autoclave at 120 °C. NaHA samples were dissolved at a concentration of 1.5-1.6 mg/mL with the carrier solution at 4 °C without vortexing in order to prevent any degradation or deformation. Prepared sample solution was stored in refrigerator for overnight to have sufficient dissolution and FI-AFlFFF/MALS experiments were made only for samples less than 10 days.

FI-AFlFFF/MALS. The FI-AFlFFF channel was the same as described elsewhere.[28,29] The depletion wall has a small inlet frit mounted inside a Plexiglass block and the length of inlet frit is 3.0 cm long from the sample inlet. The channel has a tip-to-tip length of 27.2 cm and a thickness of 178 μm which is the thickness of Mylar spacer used. The channel geometry is trapezoidal with an initial breadth of 2.0 cm and a final breadth of 1.0 cm. Both ends are cut as triangular shape. For the accumulation wall, a model PLCGC sheet membrane having molecular weight cutoff of 10 kDa which was made from regenerated cellulose by Millipore (Bedford, MA, USA) is layered.

For the delivery of carrier solution and sample, two HPLC pumps were employed: a Model 305 HPLC pump from Gilson (Villers Le Bell, France) for sample injection flow and an M930 Pump from Young-Lin Co (Seoul, Korea) for frit inlet flow. For field programming, out going cross flow was connected to the inlet of the frit flow pump with the connection of a fluid reservoir to minimize pump pulse in between and the pump outflow was directed to the frit inlet as shown in Figure 1. A needle valve was located at the end of a serial detection (MALS/DRI) to provide back pressure and regulate flow rates. Sample injection was made with a Model 7125 loop injector from Rheodyne (Cotati, CA, USA) having a 20 μL loop. The FI-AFlFFF channel outlet was connected to Dawn EOS multiangle light scattering detector from Wyatt Technology (Santa Barbara, CA, USA) with a serial connection to an Optilab DSP differential refractive index detector from Wyatt Technology for simultaneous concentration detection. The MALS used a wavelength of 690 nm. The MALS detector was set to record data from 3rd-18th angles which were 14°, 26°, 35°, 49°, 52°, 60°, 69°, 80°, 90°, 100°, 111°, 121°, 132°, 142°, 153°, and 163°, respectively. For the calibration of scattering intensity, filtered toluene (HPLC grade) was used. Albumin (BSA) was used for the normalization of MALS instrument at 90° at a flow rate of 0.10 mL/min using a Model KDS-100 syringe pump from KD Scientific (New Hope, PA, USA). The value of dn/dc was calculated as 0.165 by using the DNDC5 software from Wyatt Technology for the RI signals which were obtained by direct injection of different concentrations of HA into the an Optilab DSP interferometric refractometer. Molecular weight calculation was carried out with the software ASTRA® from Wyatt Technology. Since the accuracy in calculating molecular weight value using light scattering theory depends on how the data points measured at different angles are properly extrapolated at low angles, data points at low angles are more important for a very high molecular weight polymer than that of a small one. For the MW calculations of ultrahigh Mw NaHA in this experiment, light scattering signals from the detector numbers 4-10th only were processed with a third-order polynomial fitting by using Berry method of the Debye plot.

Results and Discussion

On-line FI-AFlFFF and MALS analysis for sodium hyaluronate samples is carried out with field programming in which cross flow rate decreases during separation. Figure

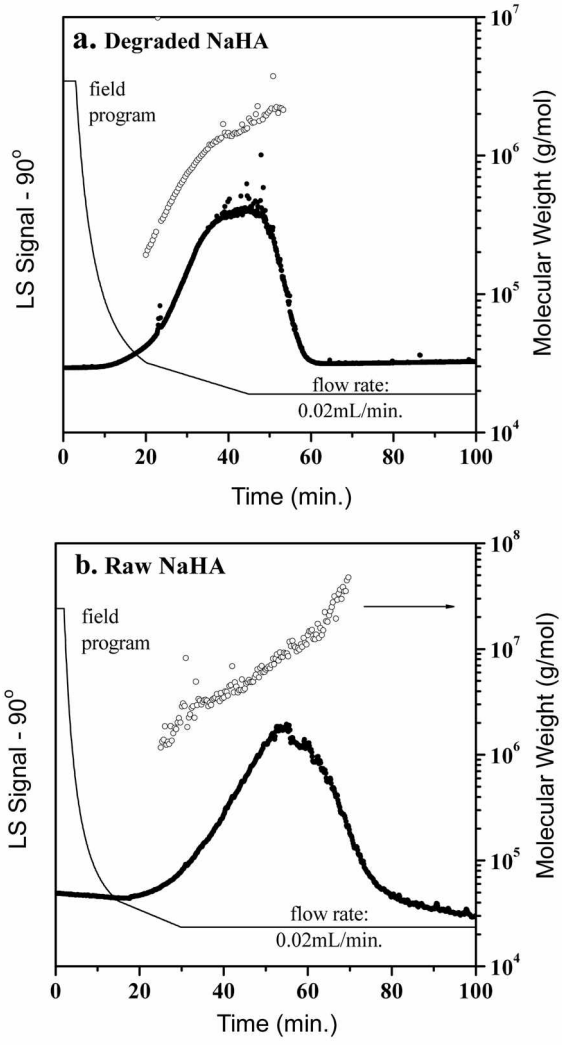

Figure 2. FI-AFlFFF fractograms (plotted with LS-90°) along with the calculated molecular weight values at each time slice of a) the thermally degraded NaHA sample and b) the raw NaHA sample. Initial cross flow rate begin with 1.0 mL/min for both runs. Decay patterns are as follows: initial delay times: a) 3 min and b) 2 min, power decay period to 0.1 mL/min: a) 17 min and b) 13 min, linear decay to 0.02 mL/min for a) 22 min and b) 15 min, final cross flow rate for both runs: 0.02 mL/min.

2 shows the FI-AFlFFF fractograms plotted with MALS signals (open circles) at 90 degree of a) the thermally degraded NaHA product and b) the raw NaHA sample eluted from an FI-AFlFFF channel. In Figure 2a the solid line represents the decay pattern of crossflow rate according to the power program with an initial cross flow-rate of 1.0 mL/min. and the initial time delay of 3 min. It decreases to 0.1 mL/min by power program for 17 min. After 20 min, it linearly decreases to 0.02 mL/min for 22 min. and then maintains at that flow rate until the end of the run. Since the crossflow is circulated to the frit flow, the frit flow rate decays simultaneously as cross flow rate does. The channel outflow rate and the sample injection flow rate are adjusted to be the same as 0.05 mL/min through the separation. The sample injection flow rate utilized in Figure 2 is 0.05 mL/min which is one-twenties of the frit flow rate (1.0 mL/min). From the earlier studies on the efficiency of hydrodynamic relaxation in FI-AFlFFF channel,[17] it was suggested that the ratio of sample flow rate to frit flow rate be kept around 0.05 to provide a sufficient relaxation. Figure 2a was redrawn from ref 12 with permission from Elsevier. In order to obtain molecular weight, the light scattering signals obtained at various angles are processed with third-order polynomial fitting by using Berry method of Debye plot. Calculated molecular weight values of the degraded NaHA sample in Figure 2a increases continuously up to 50 min which corresponds to approximately 2×10^6 Da. For the raw sample which is expected to be broader in MW distribution, a faster separation condition is selected in Figure 2b. Cross flow rate (1.0 mL/min) is maintained for 2 min of initial delay and is decayed by power program to 0.1 mL/min for 13 min. Then it linearly decreases to 0.02 mL/min for 15 min, and is fixed at this flow rate until the end of run. After adjusted to a faster field decay pattern shown in Figure 2b, separation of the raw HA sample is successfully achieved with a unimodal distribution in the molecular weight up to ~4×10^7. The raw HA sample shows some fluctuations in molecular weight values calculated at the beginning of elution which can be thought as the elution of some NaHA raw materials that are not completely relaxed. However, peak in Figure 2b clearly shows that NaHA raw materials of ultrahigh MW (> 10^6) elute at the increasing order of MW during the entire separation. This demonstrates that FlFFF technique is capable of separating high MW polymers (> 10^6 Da) without having a difficulty with packing materials often found in SEC.

Figure 3 shows a comparison of the RI fractograms of the two NaHA samples, and is superimposed together with the calculated RMS radius values at each retention time slice. During the field programming, RI baseline drifts are observed and therefore, the RI fractogram needs to be corrected by subtracting each blank run signal measured before or after a sample run using the software, Corona (v.1.40) from Wyatt Technology. Figure 3 represents that both NaHA samples elute with an increasing order of RMS radius. The calculated radius values for the two samples are clearly different from each other. The raw NaHA sample apparently shows a narrow distribution of RMS radius in the range of 100-

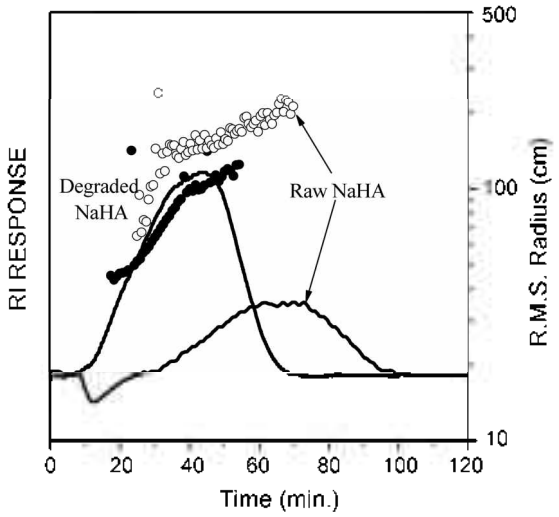

Figure 3. Comparison of RMS radius values of the two NaHA samples calculated at each retention time slice superimposed with RI signals.

200 nm while the degraded sample appears to be broader but smaller sizes (< 100 nm). The RMS radius values do not appear to be continuous between the two samples as retention time increases since the field programming condition applied for the raw sample is set to provide a faster elution of large NaHA molecules. When the RMS radius values are examined against the corresponding molecular weight values, the slope of the RMS radius against molecular weight represents conformational information regarding the molecules in solvent. Figure 4 shows a plot of RMS radius values vs. molecular weight superimposed with the cumulative weight distribution curves for both HA samples. Since the slope of the plot provides the information of fractional increase of RMS radius upon the increase of MW, it gives an idea of molecular structure in solution. The calculated slope of the degraded HA sample is 0.60 which represents a linear chain

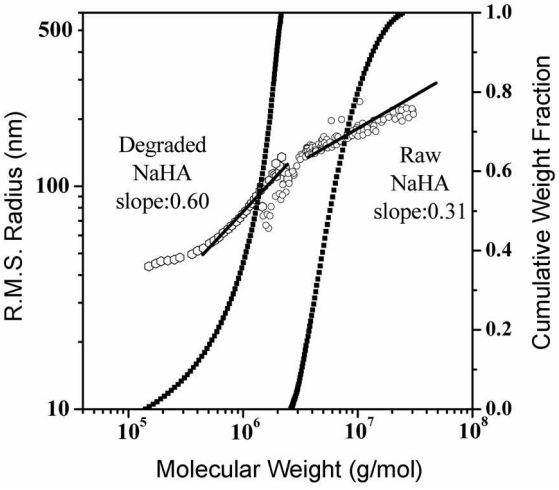

Figure 4. Plot of RMS radius vs. molecular weight for the two samples along with molecular weight distribution curve. The slope of each plot show the structure of each sample in the solution.

Table 1. The weight average molecular weight M_w, the number average molecular weight M_n, the polydisperse index M_w/M_n, and the RMS radius obtained from FI-AFlFFF/MALS experiments. Number of measurement for both samples is 3.

	The degraded HA sample	The raw HA sample
M_w (g/mol)	1.25 (± 0.27) × 10^6	7.06 (± 0.25) · 10^6
M_n (g/mol)	8.21 (± 0.56) × 10^5	4.50 (± 0.15) · 10^6
M_w/M_n	1.64 (± 0.19)	1.63 (± 0.03)
RMS radius	90.6 (± 0.7)	178 (± 1.0)

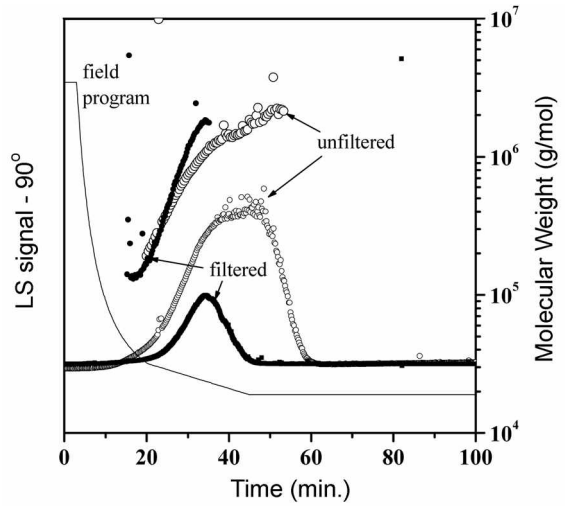

Figure 5. Effect of filtration on size distribution of the degraded NaHA sample by FI-AFlFFF/MALS.

structure (~0.66) in solution. Since the degraded HA sample is thermally degraded from the raw HA, it is expected that high molecular weight NaHA molecules are decomposed into smaller mass fractions. While the degraded HA molecules exhibit an extended structure, the calculated slope for the raw HA sample is 0.36 which is close to that (~0.33) of compact structure. The weight average molecular weight values are calculated as 1.25(± 0.09) × 10^6 (n = 3) and 7.06(± 0.25) × 10^6 (n = 3) for the degraded and the raw HA sample, respectively. Average RMS radius values for both samples are calculated to be 90.6(± 0.7) nm and 178(± 1.0) nm, respectively. These are listed in Table 1.

Figure 5 shows an influence of sample filtration on the distribution of the degraded NaHA sample before and after filtration. The degraded NaHA sample is filtered with a PVDF membrane filter having a pore size of 0.20 μm to examine the difference in molecular weight distribution. It is shown in Figure 5 that filtration causes a significant decrease in LS signals with an early completion of sample elution. In addition, most NaHA molecules larger than 10^6 Da seem to disappear during filtration. Since the injection volumes are the same for both runs, it is thought that there is a sample loss during filtration which is commonly applied for a clean up purpose prior to the separation in SEC. It is thought that a significant loss of high Mw portion of NaHA comes from the sticky nature of NaHA which is highly viscoelastic depending on the molecular weight distribution and its concentration in solution. Since separation in FFF techniques is carried out at an empty channel, sample clean up or pretreatment is not necessary to apply. This fact is particularly advantageous to perform a direct analysis of raw sample materials without inducing a loss of information such as MWD and any possible structural degradation during filtration.

Conclusions

The study demonstrates the capability of on-line FI-AFlFFF/MALS for the size characterization of ultrahigh molecular weight aqueous polymers. It is shown that FI-AFlFFF with the field programming can be utilized for the separation of broad and ultrahigh molecular weight NaHA molecules. The combination of MALS detection to FI-AFlFFF adds the analytical power in the determination of molecular weight distribution and structural information. Since coupling FlFFF with MALS does not rely on a calibration method with standards and thus, MW calculation is based on LS theory, a slight difference in FFF retention time from a possible channel contamination or flow rate variation, and etc. does not reflect to the molecular weight calculation. This is also an advantage that can be obtained when using an independent detection method. The simplicity in FI-AFlFFF operation without sample filtration is another advantage in dealing with a broad molecular weight polymeric materials. However, the long separation time in the fractionation of HA samples is a drawback of FI-AFlFFF. This can be improved to some degree by incorporating dual (field and migration flow) programming, but it brings an additional decrease in detection signal which in turn requires an increase in injection amount. Increasing sample concentration may cause another problem by inducing a possibility of sample aggregation. Further optimization of flow rates including programming condition is needed to increase separation speed without the sacrifice of resolution.

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