Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1089 (2005) 203–210
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Field programming in frit inlet asymmetrical flow field-flow fractionation/multiangle light scattering: Application to sodium hyaluronate

Heejeong Lee [a], Hoonjoo Kim [b], Myeong Hee Moon [a,*]

[a] *Department of Chemistry, Yonsei University, Seoul 120-749, South Korea*
[b] *LG life Sciences, Ltd., R&D Park, Daejeon 305-380, South Korea*

Received 21 April 2005; received in revised form 17 June 2005; accepted 27 June 2005
Available online 14 July 2005

Abstract

The capability of field-programmed separation in frit inlet asymmetrical flow field-flow fractionation (FI-AFlFFF) has been examined for separating a high molecular weight sodium hyaluronate (NaHA) by varying the field programming parameters. Experiments were performed with on-line coupling of the field programming FI-AFlFFF and multiangle light scattering (MALS) detection. Sample relaxation, a pre-requisite step to establish equilibrium states of sample materials prior to the beginning of separation in most forms of FFF techniques, is obtained by hydrodynamically in FI-AFlFFF without stopping the migration flow. Thus, the procedures of sample injection – hydrodynamic relaxation – separation in FI-AFlFFF are continuously achieved without halting the sample migration. In this study, field programming in FI-AFlFFF was investigated for the separation of NaHA, water-soluble polysaccharides, by examining the influence of field decay pattern, initial field strength condition, and ionic strength of carrier solution on the successful separation of a degraded NaHA sample. Results were compared with molecular weight calculations of eluting materials among different field programming conditions from multiangle light scattering (MALS) signals. It was found that when the field programming was utilized in FI-AFlFFF, a proper selection of initial cross-flow rate, the field decay pattern, and an appropriate control of final field strength needed to be carefully selected in achieving a successful separation of a broad molecular weight water-soluble polymer sample.

Keywords: Sodium hyaluronate; Frit inlet asymmetrical flow field-flow fractionation; Field programming; Multiangle light scattering; Molecular weight determination

1. Introduction

Flow field-flow fractionation (FlFFF) is an elution-based technique that is capable of separating and characterizing colloidal particles, proteins, and macromolecules according to the differences in hydrodynamic size of sample components [1]. In FlFFF, separation is carried out in a thin, empty channel with the use of migration flow, while cross-flow, a driving force to retain sample components within the channel, is simultaneously applied to the direction perpendicular to the axial migration flow (or channel flow) [2–5]. Before sample components begin migration, they are required to achieve equilibrium states in advance at certain distances away from the channel accumulation wall. At the equilibrium positions, the field force (from cross-flow movement) exerted to sample materials and the diffusion of sample components are counterbalanced. This process is referred as the relaxation process, which is essential in most FFF techniques and it is achieved by applying cross-flow only with the temporary halt of the migration flow. When sample components achieve equilibrium states, they are differentially distributed against the accumulation wall according to sizes and therefore, they will migrate at different velocities when axial flow is then applied. Polymers with lower molecular weight (or smaller hydrodynamic diameter) have larger diffusion coefficient val-

* Corresponding author. Tel.: +82 2 2123 5634; fax: 82 2 364 7050.
E-mail address: mhmoon@yonsei.ac.kr (M.H. Moon).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.06.069

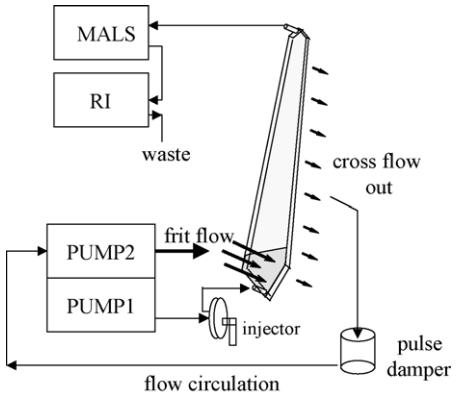

Fig. 1. Schematic diagram of frit inlet asymmetrical flow field-flow fractionation/multiangle light scattering/refractive index (FI-AFlFFF/MALS/RI) with the cross-flow circulation for programmed field operation.

ues and migrate faster at equilibrium positions that are higher than those reached by the larger ones. This is due to the differences in the flow velocities within the parabolic flow profile of the mobile phase that moves through a thin empty channel. Thus, separation in FlFFF is achieved in the order of increasing diameter or molecular weight of sample components.

Frit inlet asymmetrical flow field-flow fractionation (FI-AFlFFF) utilizes a modified channel that was designed to bypass the stop-flow relaxation procedure [5–8]. In FI-AFlFFF, sample materials are introduced through the channel inlet while a relatively high flow rate is applied through the inlet frit, as it is schematically represented in Fig. 1. Due to the compression role of the frit flow, sample materials injected at low injection flow rate (usually ca. 20 times lower than the frit flow rate) are quickly pushed toward the accumulation wall by incoming frit flow, and then they achieve hydrodynamic relaxation. Thus, sample relaxation and separation processes are continuously achieved without halting the sample migration that is normally required for the conventional FlFFF methods. While earlier studies on FI-AFlFFF channel showed that hydrodynamic relaxation can be successfully utilized to separate particles or macromolecules with a stopless flow operation, it always required the use of a high speed frit flow relative to the sample flow in order to assure sample relaxation. It might however reduce the flexibility of selecting separation conditions for highly retaining materials, such as very high molecular weight materials. The lack of flexibility can be overcome by applying field programming to FI-AFlFFF in which an initial field strength or cross-flow rate (normally set to be the same as frit flow rate) is applied sufficiently high enough to assure a successful hydrodynamic relaxation and then it is decreased gradually to fasten the elution. Field programming in FlFFF was not as popular as in other FFF techniques such as sedimentation FFF and thermal FFF but the potential of incorporating programming was already demonstrated in a few studies [4,5,9–12]. Field programming in FI-AFlFFF can be very easily employed by circulating the cross-flow to the frit flow and reducing the circulation flow rate with time. Since highly retaining components (large molecular weight) at a constant field strength condition may not elute properly when separating a broad molecular weight sample in FI-AFlFFF, a subsequent decrease in field strength during elution will be useful to elevate sample components from the vicinity of channel wall toward the fast flow streamline during run. This makes sample components eluting in a reduced time. In a previous study, it was demonstrated that programmed FI-AFlFFF can successfully expand the dynamic separation range of molecular weight utilizing polystyrene sulfonate standards in the molecular weight range of 4–1000 KDa [5].

In this study, field programming in FI-AFlFFF was applied for the separation of sodium hyaluronate, a sodium salt of hyaluronic acid, which is a natural and very high molecular weight linear polysaccharide composed of a disaccharide repeating unit (D-gluconic acid and *N*-acetyl-D-gluconsamine). NaHA has been found in various body tissues and fluids such as vitreous humour or umbilical cord, and utilized pharmaceutically for hydrogel formation or as a substitute for vitreous humor after opthalmic surgery [13–16]. The molecular weight of NaHA has been known as a few millions in Daltons, and the size characterization of NaHA in aqueous solution is important for certain desired applications. The analysis of NaHA molecules has been carried out mostly using size exclusion chromatography (SEC) [13,17]. However, when using SEC, a traditional size separation technique for polymers, difficulties in obtaining an accurate molecular weight arise from the followings such as a lack of suitable calibration standards for NaHA, a loss of resolution above the exclusion limit due to the nature of the ultra-high molecular weight of NaHA, a possible polymeric chain degradation due to the shear, and a possible sample adsorption at the surface of packing materials. Recently, a study was reported on the size characterization of NaHA by FlFFF-MALS but it was carried out with a conventional asymmetrical flow FFF channel [18]. Since the sample relaxation in the conventional asymmetrical FlFFF channel is achieved by focusing the two flows from both the channel inlet and outlet for a certain period of time, sample migration during the focusing procedure is temporarily stopped and then after the completion the separation begins by inverting the flow direction to the channel outlet. In the current work, FI-AFlFFF has been employed for the separation of high molecular weight NaHA with the use of field programming. This study focused on the evaluation of the programming conditions such as the initial field strength and field decay patterns on NaHA separation. It was also examined with the effect of ionic strength of carrier solution and injection amount on the retention of sodium hyaluronate by field programmed FI-AFlFFF. For the evaluation of separation effectiveness, on-line coupling of multiangle light scattering (MALS) detection along with refractometer (RI detector) was utilized for FI-AFlFFF and the resulting calculation in the molecular weight values or root-mean-square (RMS) radius of eluting NaHA materials were compared when varying FI-AFlFFF run conditions. By applying the field programming technique to FI-AFlFFF and on-line cou-

pling of MALS, it was demonstrated that polymers of a broad molecular weight can be well fractionated.

2. Theory

2.1. Programmed separation in FI-AFlFFF

Separation in an FI-AFlFFF channel is carried out with a stopless operation of both injection and separation procedure using hydrodynamic relaxation [6,7,19]. Once the hydrodynamic relaxation of sample components is successfully provided, sample retention in an FI-AFlFFF channel follows the basic principle of FFF found elsewhere [6].

In an FI-AFlFFF channel, the total flow rates introduced to the channel have a mass balance with the total outgoing flow rates as:

$$\dot{V}_s + \dot{V}_f = \dot{V}_{\text{out}} + \dot{V}_c \tag{1}$$

where $\dot{V}$ is the volumetric flow rate (mL/min) specified with subscripts *s*, *f*, out, and *c*, which represent sample flow, frit flow, outflow leading to detector, and cross-flow, respectively. When field programming is applied to an FI-AFlFFF channel, the frit flow rate is set to be the same as cross-flow rate so that cross-flow is circulated to the frit flow and decreased with time. In the case of using the power programming, cross-flow rate decreases as presented by the following relationship [5]:

$$\dot{V}_c(t) = \dot{V}_{c0}\left(\frac{t_1 - t_a}{t - t_a}\right)^p \tag{2}$$

where $\dot{V}_c(t)$ is the cross-flow-rate at time *t*, $\dot{V}_{c0}$ is the initial cross-flow-rate, t_1 is the initial time delay which is the period in which constant flow-rate is maintained before decay, t_a is a time parameter ($t_a = -pt_1$), and p is a power value set to 2, which is known to provide a uniform fractionating power in flow FFF [19]. The linear field (cross-flow) decay can be expressed in the following form [5]:

$$\dot{V}_c(t) = \dot{V}_{c0} - \Delta\dot{V}_c\left(\frac{t - t_1}{t_p}\right) \tag{3}$$

where t_p is the transient time for programming and $\Delta\dot{V}_c$ is the decrease in cross-flow rate during the program (that is, $\Delta\dot{V}_c = \dot{V}_{c0} - \dot{V}_c(t_1 + t_p)$). Eq. (3) is valid for $t_1 \le t \le t_1 + t_p$. For $t < t_1$, $\dot{V}_c(t)$ is fixed at initial flow rate $\dot{V}_{c0}$, and for $t > t_1 + t_p$, $\dot{V}_c(t)$ is equal to $\dot{V}_{c0} - \Delta\dot{V}_c$.

2.2. Multiangle light scattering

On-line coupling of FFF and MALS has become popular for the fractionation of water-soluble polymers and for the simultaneous measurement of absolute molecular weight, especially with asymmetrical FlFFF (or AFlFFF) [9,11,20–23]. Since the FlFFF provides size and shape based separation of macromolecules, coupling of FlFFF to multiangle light scattering measurement makes it possible to obtain Rayleigh ratio R_θ at the scattering angle (θ) in each small fraction eluting from FlFFF. The scattered light has a relationship with molecular weight according to the following equation [24]:

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

where M_w is the molecular weight, A_2 is the second virial coefficient, and K is a scattering constant containing the refractive index of solvent, and d*n*/d*c* (refractive index increment with concentration). The form factor $P(\theta)$ is to compensate for the phase difference caused by the scattering of light in different parts of macromolecules as:

$$P(\theta) = 1 + \frac{16\pi^2\langle r_G^2\rangle}{3\lambda_0^2}\sin^2\left(\frac{\theta}{2}\right) \tag{5}$$

where $\langle r_G^2\rangle^{1/2}$ is the root-mean-square radius (RMS radius). From the above equations, signals from MALS can provide calculations of molecular weight and RMS radius of each slice (volume slice) based on the consequent measurement of concentration by a refractive index detector connected in-line. This allows one to measure both molecular weight distribution and the conformational information of polymer sample by examining the molecular weight dependence of the RMS radius.

3. Experimental

3.1. Reagents

A thermally degraded NaHA sample was obtained from LG Life Sciences (Daejon, Korea). The sample was dissolved in 0.1 M $NaNO_3$ solution at a concentration of 1.6 mg/mL and 20 μL of the sample solution was injected for each injection during the all runs except the study of the influence of injection amount on separation. For the examination of injection amount, sample concentration was varied to 0.8, 1.6 and 3.2 mg/mL with an injection volume fixed at 20 μL. For the study of ionic influence of carrier solution on separation, the NaHA sample was dissolved at a concentration of 1.6 mg/mL in each different $NaNO_3$ solution (0.01, 0.05, and 0.1 M). When tested with samples dissolved in different ionic strength solutions, each corresponding solution was used as carrier solution for FI-AFlFFF separation. All carrier solutions were prepared from deionized water (>18 MΩ) containing 0.02% NaN_3 (I = 3.0 mM) as a bactericide and filtered prior to use.

3.2. FI-AFlFFF/MALS/RI

The FI-AFlFFF channel was built in-house as described in the literature [6,7]. The small inlet frit installed at the beginning end of the depletion wall has a length of 3.0 cm. The channel has a tip-to-tip length of 27.2 cm and a thickness of

178 μm, the thickness of a Mylar spacer. The channel was cut into a trapezoidal shape, which has an initial breadth of 2.0 cm and a final breadth of 1.0 cm. A regenerated cellulose membrane, PLCGC from Millipore Corp. (Billerica, MA, USA) having a molecular weight cutoff of 10 kDa, was layered at the accumulation wall.

Sample injection was made with a model 7125 loop injector from Rheodyne (Cotati, CA, USA). The sample solution was delivered to FI-AFlFFF channel by a model 305 HPLC pump from Gilson (Villers Le Bell, France) and the frit flow was delivered by a model M930 HPLC pump from Young-Lin Co. (Seoul, Korea). As shown in Fig. 1, cross-flow exiting from the channel accumulation wall was circulated to the frit flow with the use of a fluid reservoir to minimize pump pulse in between. For field programming, the frit flow rate (or cross-flow rate) was decreased during run according to field decay patterns. Eluted sample was sequentially monitored by DAWN-DSP mulitangle light scattering detector at a wavelength of 632.8 nm and an Optilab DSP interferometric refractometer (690 nm) from Wyatt Technology (Santa Barbara, CA, USA) as shown in Fig. 1. For the calibration of scattering intensity of MALS, filtered toluene was used. For the normalization of the MALS instrument, albumin (BSA) was used to detect the scattered light at 90 degree 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 of the NaHA sample used in this study was 0.165 using the DNDC5 software from Wyatt Technology from the RI signals that were measured from the direct injection of NaHA molecules by varying the concentration of NaHA under the carrier solution used in FI-AFlFFF. Data collection and molecular weight calculation were carried out with ASTRA software from Wyatt Technology. For the calculation of molecular weight and the RMS radius, the light scattering signals obtained at various angles were processed with a third-order polynomial fitting by using the Berry method of the Debye plot and the signals from the detector numbers 4–10th were used.

4. Results and discussion

4.1. Effect of programming on FI-AFlFFF separation of NaHA

The effect of field programming on the fractionation of NaHA molecules by FI-AFlFFF was examined by varying field decay pattern and the initial field strength (or cross-flow rate). Fig. 2 shows the fractograms of the degraded NaHA sample obtained at the three different field programs by varying the decay pattern and the initial delay time. Fig. 2a shows the RI signal (solid line) superimposed with the LS signal recorded at 90° (represented with filled circles) for the NaHA sample by FI-AFlFFF. The dotted line represents the decay pattern of cross-flow rate, $\dot{V}_c$, using a linear program (the program-I as listed in Table 1) with an initial delay period

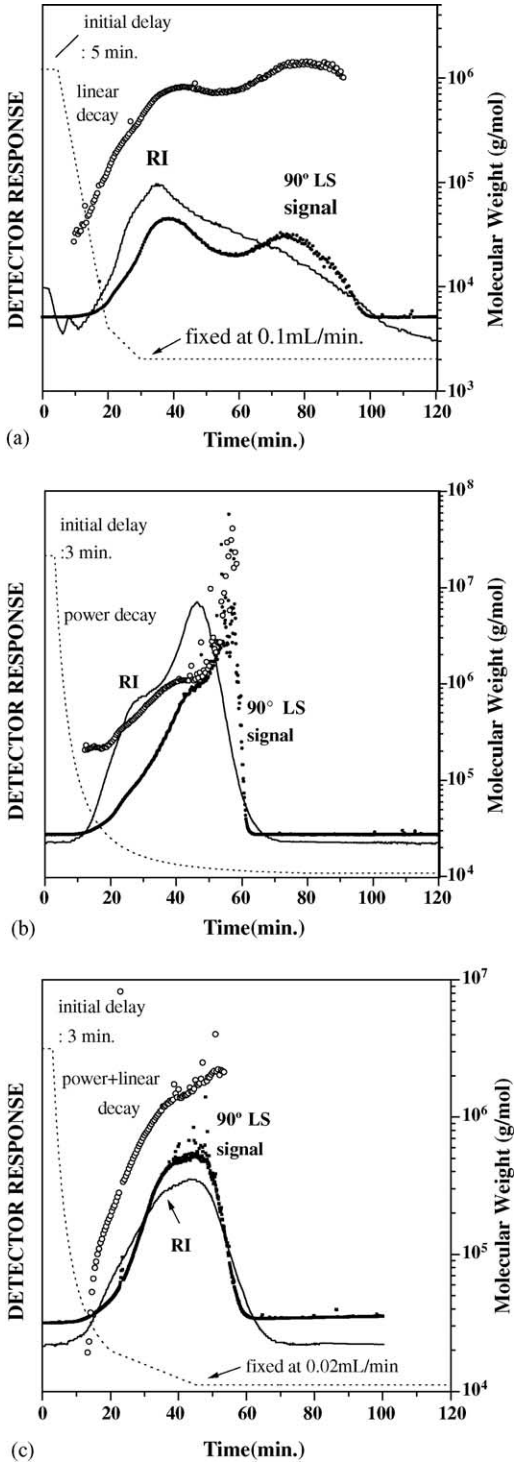

Fig. 2. Fractograms (LS-90° and RI signals) of field programmed separation of sodium hyaluronate by FI-AFlFFF at different programming parameters: (a) a linear decay (the program-I listed in Table 1); (b) a power program (the program-II); and (c) a mixed decay pattern using linear and power program (the program-III). $\dot{V}_{out} = \dot{V}_s = 0.05$ mL/min. All runs were obtained at $\dot{V}_f = \dot{V}_c$. The final field strength (or cross-flow rate) of the program-III was fixed at 0.02 mL/min from 42 min.

Table 1
Flow rate conditions, programming parameters, and the type of field programming used in this study

Program no.	Field decay pattern	t_1 pre-decay time (min)	$\dot{V}_{c0}$ intial cross-flow rate (mL/min)	$\dot{V}_{cf}$ final cross-flow rate (mL/min)	$\dot{V}_{out} = \dot{V}_s$ (mL/min)
I	Linear	5	1.0	0.1	0.05
II	Power	3	1.0	–[a]	0.05
III	Power + linear	3	1.0	0.02	0.05
IV	Power	3	2.0	–[a]	0.1
V	Linear	3	2.0	0.02	0.1

[a] Not fixed.

of 5 min. The initial cross-flow rate began with 1.0 mL/min and it decreased to 0.2 mL/min during 15 min, to 0.1 mL/min for 10 min and then it was fixed until the end of the run. Since the field programming in FI-AFlFFF was achieved by circulating the cross-flow to the inlet of the frit flow as shown in Fig. 1, the frit flow rate and cross-flow rate were adjusted to be the same and therefore the outflow rate was the same as sample flow rate, $\dot{V}_s$. The injection flow rate used in Fig. 2a was maintained at 0.05 mL/min throughout the run according to the earlier studies which suggested the use of an optimum ratio (1/20) of the sample flow rate to the frit flow rate for a successful hydrodynamic relaxation in FI-AFlFFF. During the field-programmed run, RI baseline drifts were observed. In order to compensate the baseline drifts, the current fractogram was corrected by subtracting each blank run signal measured after each corresponding sample run using the Corona software (v.1.40) from Wyatt Technology. While the RI signals of eluted NaHA in Fig. 2a showed a broad distribution with a small shoulder at around 75 min of retention, the LS signal showed a distinct bimodal distribution. The difference in the signal intensities between RI and MALS originates from the fact that the light scattering detection is more sensitive for large molecular weight components than a concentration detector such as RI. Molecular weight values were calculated at each volume slice of the fractogram by extrapolating the LS signals at different angles using ASTRA software and they were superimposed as open circles in Fig. 2a. As retention time increases, it appeared that molecular weight value increases continuously but there appears with a slow increase in the middle of apparent bimodal peak. If there is a distinct bimodality in the distribution, the increase of molecular weight values should be constistent. However, it was not clear to determine with this data alone whether the real distribution of the NaHA sample used in this study was bimodal or it was induced by an inadequate selection of field decay pattern. The same sample was examined with a power program (the program-II) given in Eq. (2) and the fractogram was shown in Fig. 2b obtained at the same intial $\dot{V}_c$ but at a reduced initial delay time ($t_1 = 3$ min). Sample flow and outflow rate were kept the same as used in Fig. 2a. Due to the relatively fast decay of field strength in power programming caused by a shorter initial delay period, it appeared that late eluting components of the NaHA sample shifted to the shorter time scale but the LS signals were scattered at the peak maximum. Fluctuations in the LS signals supported that the size fractionation of NaHA molecules at the peak maximum was not properly made. However, the fast and continuous decrease of field strength was found to be useful to shorten retention. The molecular weight values plotted in Fig. 2b showed that there was a fluctuation in the calculated values. It supported that there was a serious loss of resolution after the peak maximum point due to the sudden loss of field strength. When it was used with a longer initial delay time (5 min), field decay pattern was relatively shallow and elution peak was extended with a broader distribution (the result was not shown here) as it was observed in Fig. 2a. From the variation of decaying pattern, a modified field program using the combination of power decay and linear decay patterns was selected with the minimum field strength fixed at a certain level of flow rate as shown in Fig. 2c. The decay pattern used in Fig. 2c began with the power program as used in Fig. 2b until 20 min (0.1 mL/min up to this time), and it decreased linearly to 0.02 mL/min for 25 min, and then it was maintained at 0.02 mL/min until the end of run. The incorporation of the fixed final cross-flow rate was helpful to keep sample components from being swept abruptly when field strength reached to nearly zero. The lower limit used in Fig. 2c was selected from the variation of different minimum flow rates. Fig. 2c showed a continuous increase in molecular weight values according to the increase of retention time. From Fig. 2c, it can be thought that the apparent bimodal distribution observed at Fig. 2a was induced from the difference in field decay patterns. The weight average molecular weight values were 0.79×10^6 for the program-I and 1.15×10^6 obtained for the program-III. Due to the severe scattering in the calculation, the average value for the run obtained from the program-II was not meaningful to include here.

In Fig. 3, a higher cross-flow rate (equal to frit flow rate in programmed FI-AFlFFF) was employed to check any difference in FI-AFlFFF retention of the NaHA sample. Since an increase of frit flow rate in an FI-AFlFFF channel also increases the movement of effective migration flow, separation can be achieved without incurring a significant increase in retention time even though the cross-flow rate was simultaneously raisd. In addition, using a higher frit flow rate can allow one to use an increased sample injection flow rate as well as outflow rate. Fig. 3 showed the comparison of elution profiles of the NaHA sample between the two programmed decay patterns; a power decay for the program-IV (expressed

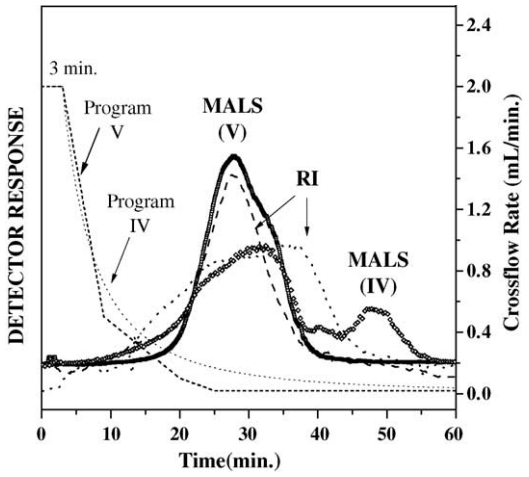

Fig. 3. Superimposed fractograms of NaHA obtained at two different decay patterns (the program-IV (power decay) and -V (linear decay)) by FI-AFlFFF. The initial cross-flow rate, $\dot{V}_{c0}$, was 2.0 mL/min and $\dot{V}_{out} = \dot{V}_s = 0.1$ mL/min. The final field strength of the program-V was fixed at 0.02 mL/min from 25 min.

as dotted line) and a linear decay for V (dashed line). Both runs used the same initial cross-flow rate at 2.0 mL/min and the initial delay period of 3 min. In order to meet the requirement of the ratio (1/20) of the injection flow rate to the frit flow rate in FI-AFlFFF, sample injection in Fig. 3 was made at 0.1 mL/min along with the simultaneous increase of the outflow rate which was doubled from the value used in Fig. 2. Peaks obtained from both runs were represented with RI signals and LS signals simultaneously. By utilizing a higher frit flow rate and outflow rate, separation speed was greatly improved. However, when the program-IV was utilized, retention of the NaHA sample appeared with a bimodal distribution in LS signal as it was observed with the program-I while the RI signal showed a broad but nearly unimodal distribution. A serious splitting of distribution disappeared when the program-V was employed. The decay pattern of the program-V followed the three stages of linear decrease in cross-flow rate (listed in Table 1) and it was fixed at the same lower limit of final cross-flow rate, 0.02 mL/min, from 25 min. Except a small shoulder in the LS signal obtained by the program-V, separation appeared to be made without being splitted throughout the run. It was noted that light scattering detection of the high molecular weight NaHA sample was sensitive to the slight difference in the field decay patterns.

In order to evaluate the efficiency of size fractionation of the NaHA sample between the two field decaying programs (III and V), the calculated molecular weight values were plotted against elution time along with LS-90° signals in Fig. 4a and they were compared in cumulative scale in Fig. 4b. Both calculations showed that molecular weight value of each slice increased with the increase of retention time. This observation supported that the fractionation of NaHA by programmed separation of FI-AFlFFF was accomplished with an increasing order of molecular weight value. When comparing size calculations, both runs showed similar molecular weight

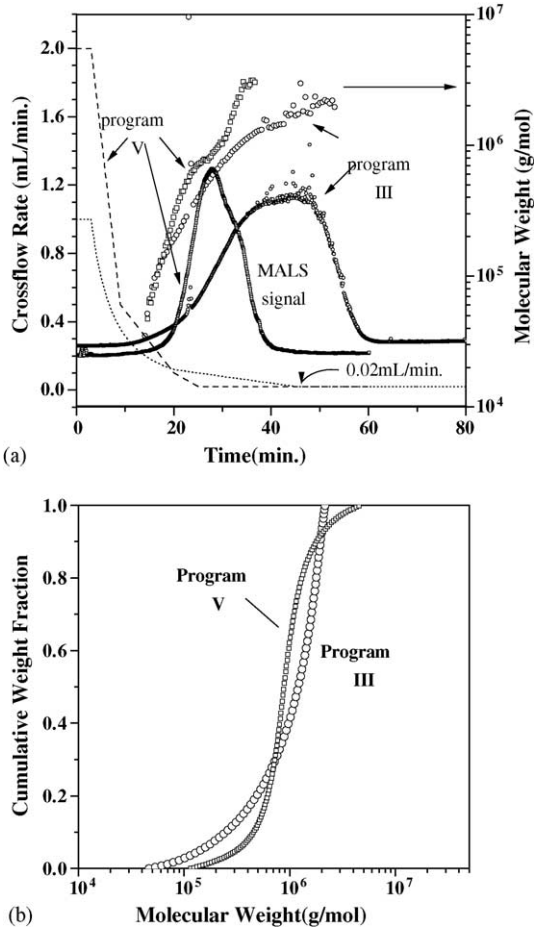

Fig. 4. (a) Comparison of the LS signals obtained at two different flow rate conditions (the program-III and -V) along with the calculated molecular weight values at each retention time slice and (b) the cumulative molecular weight distribution curves for NaHA obtained at two different run conditions.

values at the upper limit, and the weight average molecular weight value was calculated as 1.15×10^6 obtained for the program-III and 1.04×10^6 for the program-V. The polydispersity values for both runs were 2.09 and 1.42, respectively. Both run conditions appeared to provide similar average MW values, but an advantage of using the higher initial frit flow rate and higher outflow rate condition yielded with a faster separation of the NaHA sample as well as with an efficient fractionation according to the MWD curve shown in Fig. 4b. The recovery values were found to be $83.3 \pm 13.1\%$ ($n = 3$) and $87.1 \pm 13.1\%$ ($n = 4$) for the runs from the program-III and -V, respectively. The recovery value was based on the comparison of the LS signal area between runs obtained with and without applying the field strength. The recovery values were similar to those reported for FI AFlFFF system [25]. From the above experiments, it was found that the field programming technique in FI-AFlFFF channel demonstrated the capability of separating a broad molecular weight range about 2 orders of magnitude. In case of using a constant field strength in a typical flow FFF run, it often encountered with a certain limitation in molecular weight range of separation.

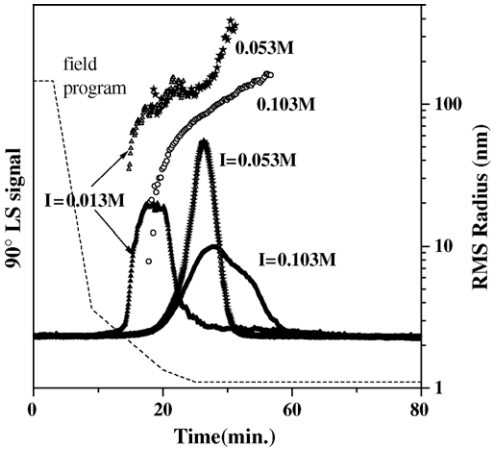

Fig. 5. The effect of ionic sterngths of the carrier solution ($NaNO_3$ with 0.02% NaN_3) on FI-AFlFFF separation of the degraded NaHA and on the calculated values of RMS radius at each slice. The ionic strength of carrier solution was varied by changing concentration of $NaNO_3$ from 0.01 to 0.1 M). The run condition used was the program-V.

However, the results in Fig. 4 provided that the molecular weight calculations were consistently made without being affected from the difference in separation conditions once they were fractionated properly in FI-AFlFFF.

4.2. Effect of ionic strength

The behavior of macromolecules in solution is complicated due to the molecular interaction caused by electrostatic forces, and it is related with a proper selection of salt concentration of polymer solution that determines an accurate size and conformation information of aqueous polymers. Ionic strength of carrier solution used in FlFFF separation is an important parameter when separating aqueous polymers since it will influence the electrostatic interactions among NaHA molecules or the interaction between HA molecules and the channel wall. The intramolecular electrostatic repulsion may also change the hydrodynamic size of HA molecules in solution. This will eventually result in the change of retention time in FlFFF. In order to study the effect of ionic strength of carrier solution on the retention of NaHA, field-programmed separation of NaHA was performed at three different concentrations (0.01, 0.05, and 0.1 M) of $NaNO_3$ solution. The total ionic strength values of each solution including NaN_3 (=0.003 M) were $I = 0.013$, 0.053, and 0.103 M.. The sample solution was separately prepared by dissolving the NaHA sample at each corresponding carrier solution. Fractionation of the NaHA sample was carried out by applying the program-V as utilized in Fig. 4. Fig. 5 compared the different LS fractograms obtained at the different ionic strengths along with the calculated RMS radius values from the LS signals. The peaks showed that there was a significant difference in the retention time and the distribution of NaHA as ionic strength varies. When the ionic strength of the carrier solution increased from $I = 0.013$ to 0.103 M, the retention of NaHA was extended to a longer time scale. Since the electrical double layer formed at the channel wall decreased as the ionic strength of carrier solution increased, it can be thought that NaHA molecules migrated closer to the channel wall at a carrier solution of a higher ionic strength and thus, they retained longer. In considering the hydrophobic interactions among NaHA molecules caused by the difference in ionic strengths, it was helpful to compare the RMS values calculated from MALS data obtained at the different ionic strength conditions. When comparing the calculated RMS values between $I = 0.053$ M and $I = 0.103$ M, the NaHA sample began eluting at nearly the same time (∼20 min), but the RMS values obtained from the lower ionic strength condition (represented as filled star symbols) appeared to be higher than those from the higher ionic strength condition (open circles). This represented that NaHA molecules migrated at more elevated positions away from the channel wall at a lower ionic strength. The electrical double layer at a lower ionic strength solution became more diffuse at the channel wall and this can increase the equilibrium heights of sample migration, which in turn fasten the elution. The current study was focused on the optimization of NaHA separation in FI-AFlFFF, details on the structure and the size of NaHA molecules was not thoroughly examined in this article. However, the calculated RMS values did not increase smoothly as retention time increased except the run condition of $I = 0.103$ M. It supported that 0.1 M $NaNO_3$ solution appeared to be suitable for the separation of NaHA by field programmed FI-AFlFFF.

4.3. Effect of injection amount

Influence of injection amount on the retention of NaHA molecules was examined by varying the sample concentration but at a fixed volume (20 μL) of injection. The purpose of using a fixed injection volume was to keep from inducing any band broadening caused by the difference in the injection periods. In case of using a very low sample flow rate in an FI-AFlFFF channel, an additional band broadening may occur when time period of injection differs by varying injection volume. Fig. 6 was obtained by injecting NaHA solutions varied at three different sample concentrations: 0.8, 1.6, and 3.2 mg/mL. The flow rate conditions were the same as used in Fig. 4 with the program-V. Fig. 6 showed a similar elution profile that was observed without a distinct shift of retention time when injected amount of NaHA was doubled from 16 μg (0.8 mg/mL) to 32 μg. In case of 32 μg injected, it was not significantly different from the repeated run. However, when 64 μg of NaHA was injected at a concentration of 3.2 mg/mL, the NaHA peak maximum time and its distribution shifted toward a little longer time scale. In addition, the peak intensity did not increase correspondingly as it was expected. It can be explained with possibilities such as the aggregation of NaHA molecules or the poor solubility of NaHA molecules in a more concentrated sample solution. The first one may result in the shift of retention time and the second may lead to an insufficient sampling by microsyringe. Experimentally,

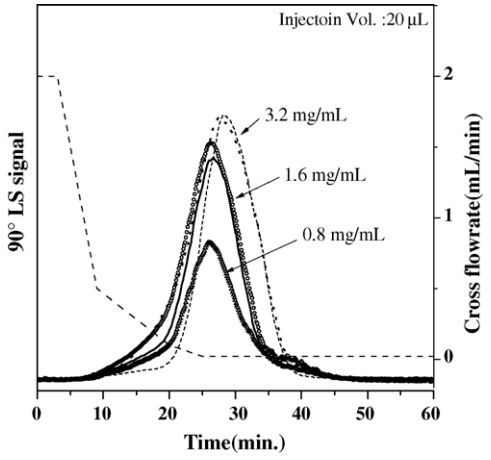

Fig. 6. Influence of sample injection amount on retention of the NaHA sample by FI-AFlFFF. Injection volume was fixed at 20 μL with the variation of sample concentrations as 0.8, 1.6, and 3.2 mg/mL. The run condition was the same as used in Fig. 5 at $I = 0.103$ M.

it was difficult to withdraw the highly concentrated sample solution by microsyringe since the sample solution was too viscous. The difficulty in sampling may cause an insufficient injection of the sample molecules. From these evaluations, the sample concentration higher than 1.6 mg/mL was found to be inappropriate in sampling such a viscous solution and for a reproducible work.

5. Conclusions

In this work, capabilities of field-programmed separation in FI-AFlFFF were demonstrated with a sodium hyaluronate sample by optimizing field programming parameters along with other experimental considerations. It was found that a proper selection of initial cross-flow rate and an appropriate selection of decay pattern with the control of final field strength were all important in achieving a successful separation of a broad molecular weight NaHA sample in FI-AFlFFF. Moreover, other experimental parameters such as ionic strength of carrier solution, injection amount, and sample concentration were critical in FI-AFlFFF separation of NaHA. Since separation in FI-AFlFFF was carried out by hydrodynamic relaxation without stopping sample migration after injection, it can minimize a fear of dealing with some polymeric materials that can easily adhere on channel wall under a very high field strength. In case of separating a broad molecular weight polymer sample that ranges up to ultra-high MW, a hydrodynamic relaxation technique with field programming may be conducive to expand the dynamic separation range of MW and to retrieve long retaining sample component.

Acknowledgements

This study was supported by Korea Research Foundation Grant (KRF-2004-015-C00348). The authors wish to thank P. Reschiglian, University of Bologna, for his fruitful discussion.

References

[1] J.C. Giddings, Science 260 (1993) 1456.
[2] J.C. Giddings, Anal. Chem. 53 (1981) 1170A.
[3] K.-G. Wahlund, J.C. Giddings, Anal. Chem. 59 (1987) 1332.
[4] S.K. Ratanathanawongs, J.C. Giddings, Anal. Chem. 64 (1992) 6.
[5] M.H. Moon, D. Kang, I. Hwang, P.S. Williams, J. Chromatogr. A 955 (2002) 263.
[6] M.H. Moon, H.S. Kwon, I. Park, Anal. Chem. 69 (1997) 1436.
[7] M.H. Moon, P.S. Williams, H. Kwon, Anal. Chem. 71 (1999) 2657.
[8] D.J. Kang, M.H. Moon, Anal. Chem. 76 (2004) 3851.
[9] B. Hecker, P.D. Fawell, A. Jefferson, J.B. Farrow, J. Chromatogr. A 837 (1999) 139.
[10] K.-G. Wahlund, H.S. Winegarner, K.D. Caldwell, J.C. Giddings, Anal. Chem. 58 (1986) 573.
[11] H. Thielking, D. Roessner, W.-M. Kulicke, Anal. Chem. 67 (1995) 3229.
[12] M. Andersson, B. Wittgren, H. Schagerlof, D. Momcilovic, K.-G. Wahlund, Biomacromolecules 5 (2004) 97.
[13] Z. Iqbal, J.M. Midgley, D.G. Watson, S.D. Karditsas, G.N. Dutton, W. Wilson, Pharm. World Sci. 19 (1997) 246.
[14] C. Yeung, D. Marecak, J. Chromatogr. A 852 (1992) 573.
[15] K.P. Vercruysse, G.D. Prestwich, Crit. Rev. Ther. Drug Carrier Syst. 15 (1998) 513.
[16] N. Motohash, Y. Nakamichi, I. Mori, H. Nishijawa, J. Umemoto, J. Chromatogr. 435 (1988) 335.
[17] H. Sasari, T.Y. Konttinen, S. Santavirta, Med. Sci. Res. 18 (1989) 99.
[18] R. Takahashi, S. Al-Assaf, P.A. Williams, K. Kubota, A. Okamoto, K. Nishinari, Biomacromolecules 4 (2003) 404.
[19] P.S. Williams, J.C. Giddings, Anal. Chem. 59 (1987) 2038.
[20] B. Wittgren, K.-G. Wahlund, J. Chromatogr. A. 760 (1997) 205.
[21] W. Fraunhofer, G. Winter, C. Coester, Anal. Chem. 76 (2004) 1909.
[22] L. Picton, I. Bataille, G. Muller, Carbohydr. Polym. 42 (2000) 23.
[23] C. Duval, D.L. Cerf, L. Picton, G. Muller, J. Chromatogr. B. 753 (2001) 115.
[24] P.J. Wyatt, Anal. Chim. Acta 272 (1993) 1.
[25] M.H. Moon, I. Hwang, J. Liq. Chromatogr. Rel. Technol. 24 (20) (2001) 3069.