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Molecular weight and structure characterization of sodium hyaluronate and its gamma radiation degradation products by flow field-flow fractionation and on-line multiangle light scattering

Da Young Shin [a], Euijin Hwang [b], Il-Hwan Cho [b], Myeong Hee Moon [a,*]

[a] *Department of Chemistry, Yonsei University, Seoul 120-749, South Korea*
[b] *Department of Biotechnology, Shinpoong Pharmaceutical Co. Ltd., Ansan, Kyeonggi-Do, South Korea*

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Abstract

A combined flow field-flow fractionation (FlFFF)/multiangle light scattering (MALS)/differential refractive index (DRI) detection method has been utilized for the size fractionation and characterization of ultrahigh molecular weight (MW) sodium hyaluronate (NaHA) samples. Separation of broad MW NaHA polymers was carried out by a frit inlet asymmetrical FlFFF channel employed with a linear field programming method followed by the on-line monitoring of light scattering at multiple angles for the calculation of MW and for the study of the conformation of NaHA samples. NaHA samples examined were: (1) two different viscosity fractions of NaHA obtained by a refinement process and (2) NaHA products of gamma radiation degradation. While the NaHA samples of two different viscosity fractions exhibited clearly different MW distributions and similar conformation, the radiation degraded NaHA samples showed a clear difference in both MW distribution and polymer structure.

Keywords: Flow field-flow fractionation; FFF; Gamma radiation; Sodium hyaluronate; Molecular weight distribution; Conformation of sodium hyaluronate; Field programming

1. Introduction

Characterization of the size and structure of macromolecules is an important task of polymer science in relation to studying material properties during synthesis, purification and processing. Among various polymers, water soluble polymers are of interest in pharmaceutical applications. Sodium hyaluronate (NaHA), a polysaccharide having a disaccharide repeating unit (D-glucuronic acid and *N*-acetyl-D-glucosamine), is an ultrahigh MW water soluble polymer which can be found in body tissues, synovial fluid, the vitreous humor, the umbilical cord, and elsewhere [1–5]. Intact or degraded forms of this material have been utilized in the cosmetic industry, in ophthalmic surgery, for drug delivery and for the treatment of knee joint disease [6,7].

Size exclusion chromatography (SEC) with spectrometric or viscometric detection techniques has been widely utilized for the separation and characterization of soluble polymers [8]. However, for ultrahigh molecular weight (MW) components (>a few million g/mol), using conventional SEC columns is unsuitable due to the limitation of pore sizes, a possibility of a shear-induced degradation of large MW components, and column blocking.

Alternatively, a method that has been used for the separation of ultrahigh MW materials is flow field-flow fractionation (FlFFF). The FlFFF technique is capable of fractionating colloidal particles and large macromolecules such as polymers, proteins and DNA by the differences of hydrodynamic diameter and shape [9–13]. In the case of spherical components, FlFFF also provides an accurate theoretical calculation of hydrodynamic sizes of sample components [10]. When it is hyphenated on-line with multi-angle light scattering (MALS) along with a concentration detector such as refractive index (RI) or UV detector, it is possible to obtain molecular weight value of an eluted sample component, the molecular weight distribution of polymers and conformational information. The on-line FlFFF-MALS-DRI has been utilized for the characterization of broad MW polymeric materials such as polyacrylamide [14], modified cellulose [15,16], polysaccharide gum Arabic [17] and pullulan [18]. The separation and characterization of NaHA materials

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

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using on-line FlFFF-MALS was first attempted by Takahashi et al. [7] utilizing an asymmetrical FlFFF (AFlFFF) channel. More recently it was studied by Moon and co-workers [19–21], who employed the frit inlet asymmetrical FlFFF (FI-AFlFFF) channel in which sample injection and separation can be achieved continuously without halting the migration flow [22–24]. In our recent work [21], the effect of sample dissolution temperature on the size and conformation of NaHA was examined with FlFFF/MALS/DRI.

In this study, the molecular weight distribution of two NaHA materials of different viscosity grades was examined using on-line FlFFF-MALS-DRI. The combined method was applied to: (1) monitor the efficiencies of the refinement process and (2) measure the effect of gamma radiation degradation on the MW distribution and conformation of NaHA molecules. To enhance the fractionation capability of FlFFF for the broad and ultrahigh MW NaHA samples, linear programming of the crossflow field was employed in an FI-AFlFFF channel.

2. Experimental

2.1. Materials and reagents

All NaHA samples were provided by Shinpoong Pharm. Co. Ltd. (Ansan, Korea). These samples were extracted from fowl sarcoma fluid and were processed for commercial usage. High and low viscosity fractions of NaHA samples were obtained by a proprietary process from Shinpoong Pharm. Co. Ltd. Degraded NaHA samples were obtained from Shinpoong Pharmaceutical Co. Ltd. after gamma ray irradiation of a raw NaHA material with doses of 2.3, 5.3 and 7.4 kGy (hereafter expressed with 2, 5 and 7 kGy) using an Amber 3042 Dosimeter from Harwell Dosimeters Ltd. (Oxfordshire, UK). Different doses of radiation were obtained by the different time period of exposure as 3 h (2.4 kGy), 8 h (5.3 kGy) and 10 h (7.4 kGy). For the dissolution of NaHA, samples were dispersed in a 0.1 M $NaNO_3$ solution containing 0.02% NaN_3 as bactericide, which was found from an earlier evaluation on the effect of ionic strength of solution on the intermolecular electrostatic interaction [19]. NaHA samples were dissolved at a concentration of 0.8–1.0 mg/mL with carrier solution at 4 °C overnight without stirring in order to prevent any degradation. Prepared sample solution was stored in refrigerator and a volume of 20 μL was injected for all runs. The $NaNO_3$ solution was used as the carrier solution in FlFFF separation. This carrier solution was prepared with deionized water (>18 MΩ cm) and filtered with a membrane filter having a pore size of 0.02 μm prior to use.

2.2. FlFFF/MALS/DRI

The FlFFF channel utilized in this study was a frit inlet asymmetrical FlFFF channel, details of which are provided in earlier reports [19,21]. The FI-AFlFFF channel space was made with a 178 μm thick Mylar spacer by cutting the center region into the following dimensions: a tip-to-tip length of 27.2 cm, an initial breadth of 2.0 cm decreasing trapezoidally to a final breadth of 1.0 cm. Both ends of the trapezoidal channel space were cut in a triangular shape. The geometrical channel volume was 0.70 mL. The FI-AFlFFF channel was equipped with a small inlet frit (3.0 cm from the sample inlet) at the depletion wall of the channel. A high speed flow stream (frit flow) was delivered through this frit to provide a hydrodynamic relaxation of the sample components entering the channel with the sample injection stream. At the accumulation wall of channel, a regenerated cellulose membrane (PLCGC with 10,000 MWCO, from Millipore Corp., Billerica, MA, USA), was placed to keep the sample components from penetrating through the frit by the movement of crossflow. In this study, the investigation of a possible interaction between NaHA and the membrane was not included.

The FI-AFlFFF channel was connected with two HPLC pumps; one for the delivery of the sample, a Model 305 HPLC pump from Gilson (Villers Le Bell, France), and the other for the frit flow, a Model M930 HPLC pump from Young-Lin Co. (Seoul, Korea). For the field programmed separation in FI-AFlFFF, crossflow out was circulated by connection to the inlet of the frit flow pump and its flow rate was decreased according to the programmed pattern. With this configuration, the crossflow field can be decreased during FI-AFlFFF separation of NaHA materials. A Rheodyne injector, a Model 7125 loop-type from Rheodyne (Cotati, CA, USA) with 20 μL loop volume was placed between the sample flow pump and the channel inlet. At the end of the channel outlet, a DAWN-DSP multiangle light scattering detector at a wavelength of 632.8 nm and an Optilab DSP differential refractive index (DRI) detector at a wavelength of 690 nm from Wyatt Technology (Santa Barbara, CA, USA) were connected in sequence. At the end of the DRI detector, a syringe pump, Model PN1610 Syringe Dosing System from Postnova Analytics (Lansberg, Germany), was placed in suction mode to remove the outflow at a constant flow rate. This was helpful to maintain a consistent outflow rate during the programmed crossflow rate decrease. For the calibration of the MALS instrument, filtered toluene was used. For the normalization of the MALS instrument, albumin (BSA) was used to detect the scattered light at 90° at a flow rate of 0.10 mL/min with a model KDS 100 syringe pump from KD scientific (New Hope, PA, USA). The dn/dc of NaHA was measured with an Optilab DSP interferometric refractometer with respect to concentration variation; this calculation was made using DNDC5 software from Wyatt Technology. Calculated values of dn/dc for the NaHA samples were 0.142 and 0.211 for low and high viscosity samples, respectively, and 0.167 for the NaHA sample treated for radiation degradation. For the calculation of molecular weight, LS signals of the angles (4–10th) were processed with ASTRA software from Wyatt Technology using a third-order polynomial fit according to the Berry method of the Debye plot as suggested in the literature [25]. Since the signals at the third angle were so fluctuating when detected for ultrahigh MW components, they were not included for the MW calculation. Baseline correction of each DRI fractogram was applied with each blank run due to the shift of the detector baseline during field programming and it was done by CORONA software from the manufacturer. DRI signals were corrected for the inter-detector time delay (inter-detector volume as 0.12 mL) by the software.

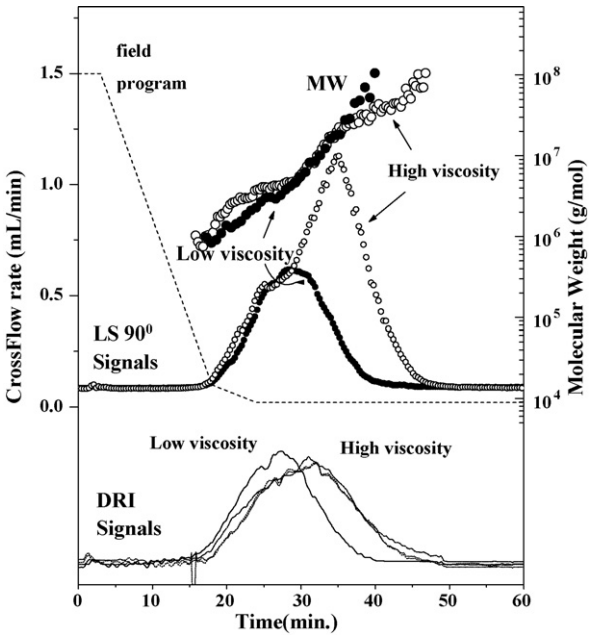

Fig. 1. FlFFF fractograms (DRI signals at the bottom and MALS at 90°) of NaHA samples of different viscosity fractions and MW values calculated at each retention time slice. Flow rate conditions are as follows: injection flow rate and outflow rate were fixed at 0.09 mL/min, crossflow and frit flow were linearly decreased from 1.5 to 0.02 mL/min by the pattern illustrated by the dotted line.

3. Results and discussion

FlFFF/MALS/DRI analyses of the two NaHA samples of different viscosities are presented in Fig. 1. These samples were run at the same crossflow field programming condition, shown in Fig. 1 with a dotted line. Initial crossflow rate began with 1.5 mL/min for 3 min and then decreased linearly to 0.1 mL/min over 15 min, then decreased again to 0.02 mL/min over 6 min and then, finally, was maintained at 0.02 mL/min until the end of separation (the flow rate scales are provided on the left axis of Fig. 1). Since an FI-AFlFFF channel was utilized for the field programmed separation of NaHA in this study, the frit flow rate was adjusted to be the same as the crossflow rate for circulation and the channel outflow rate was the same as the injection flow rate of 0.1 mL/min. In the superimposed fractograms (LS signals of 90°) of the two NaHA samples shown at the top of Fig. 1, the two different viscosity NaHA samples eluted at similar retention times (after 15 min), but the high viscosity sample eluted with a broader peak. The injection amount of each sample was 20 and 16 μg for the low and high viscosity NaHA samples, respectively. While the injection amount of the high viscosity sample was less than that of the low one, the LS fractogram of the higher viscosity sample showed a stronger intensity at the late-eluting part of the peak. When the DRI signal intensity (lower part of Fig. 1) of the high viscosity sample (represented with the three fractograms from repeated runs) is compared with the LS signals for the high viscosity sample, a significant difference in intensity is apparent. This difference is due to the fact that light scattering depends on both MW and concentration whereas the refractive index only depends on sample concentration. DRI signals shown are corrected for inter-detector time delay.

With reference to the elution order of FlFFF separation, the high viscosity sample appeared to be larger and broader in molecular weight. MALS signals recorded at each angle during the elution of NaHA were utilized for the calculation of MW at each retention time slice. MALS signals along with the DRI signal (for concentration information) and the resulting MW values are plotted in Fig. 1 using filled (low viscosity sample) and open (high viscosity sample) circles. The MW scale is logarithmic and is shown on the right axis. Except for the slight difference in the lower MW region (1×10^6 to 4×10^6 g/mol), MW values of the two different viscosity samples increased steadily as retention time increased. This indicates that FlFFF separation of NaHA molecules was carried out successfully with increasing MW. For the high viscosity NaHA, MW values were calculated to be quite large, up to 1×10^8 g/mol.

The resulting MW distribution (MWD) curves are plotted in Fig. 2a and show a significant difference in weight average MW (Mw) of 4.2 (±0.05) $\times 10^6$ and 9.5 (±0.1) $\times 10^6$ ($n = 4$) for the

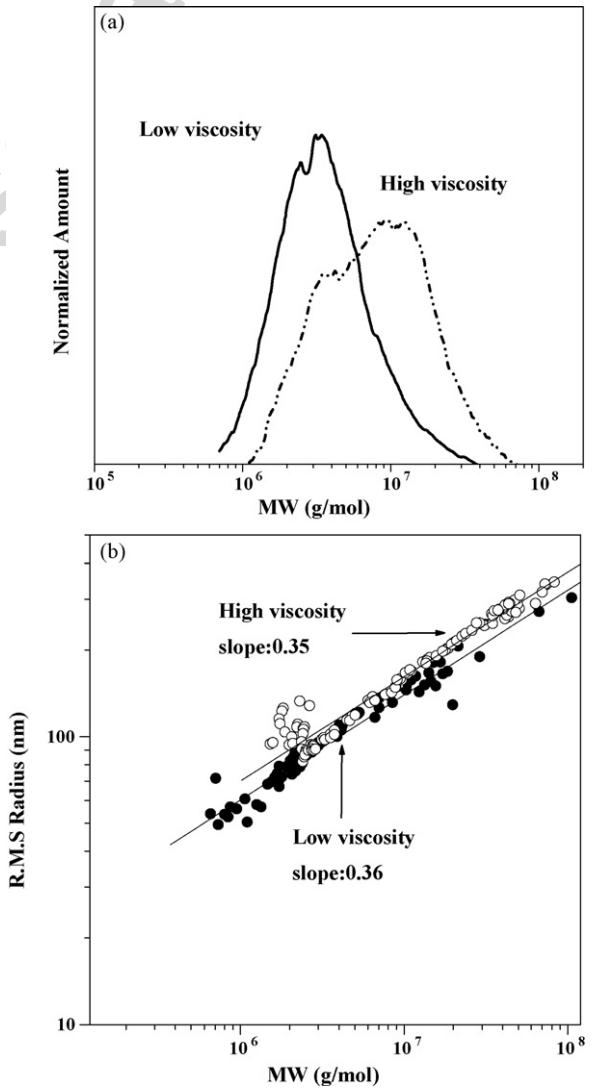

Fig. 2. (a) Comparison of the MWD curves (normalized) between the two different viscosity fractions of NaHA polymers and (b) plots of RMS radius vs. MW.

Table 1
Molecular weight, RMS radius values and RSD ($n = 4$) of the two NaHA samples with different viscosities by FI-AFlFFF/MALS/DRI

	Low viscosity sample	High viscosity sample
Intrinsic viscosity (m^3/kg)	2.2	3.2
Mw (g/mol)	$(4.2 \pm 0.05) \times 10^6$	$(9.5 \pm 0.1) \times 10^6$
Mn (g/mol)	$(2.8 \pm 0.03) \times 10^6$	$(5.0 \pm 0.05) \times 10^6$
Mw/Mn	1.50 ± 0.03	1.88 ± 0.03
RMS radius (nm)	108.7 ± 0.9	195.6 ± 0.8

low and high viscosity samples, respectively. The number average MW (Mn), polydispersity, and RMS radius values of the two NaHA samples are listed in Table 1. The structures of the two NaHA samples can be inferred using a plot of the root-mean-square (RMS) radius values plotted against their corresponding MW values (Fig. 2b). This figure shows that the plots of the two samples are superimposed in a common region where the slope values of the regression are similar to each other (~0.36). This similarity indicates that they have a rather compact molecular geometry, while MWD curves of the two samples (in Fig. 2a) are significantly different from each other. The common conformations of NaHA in the solid state were reported to be a fully extended molecule or a multiply folded helix stabilized by hydrogen bonds between glycosidic linked monomers [5]. In our earlier experiment [21], we reported that as is evidenced by the high dissolution temperature for NaHA, these materials may undergo a transformation toward a less entangled or linear structure from a compact and folded geometry. This conclusion was made by noting the difference in slope values (from 0.34 to 0.65). In this case, since the slope values (Fig. 2b) are similar it is concluded that the difference in the viscosity values of the two samples originates from the difference in the MWDs and is not related to specific molecular structures.

FlFFF/MALS/DRI was further applied for the evaluation of the effect of gamma radiation degradation on MWD and conformation of NaHA molecules. Since gamma radiation degradation of polymers is known to reproducibly effect a substantial change in MWD by hydrolysis without using chemicals [26,27], it was utilized to develop a process to obtain different MWD fractions of NaHA. Fig. 3 shows the FlFFF/MALS/DRI results of a NaHA control sample and the three NaHA products made by degradation of the control sample using gamma radiation at different exposure periods. The flow rate conditions employed in Fig. 3 were slightly modified from those used in Fig. 1. While the injection flow and outflow rates were fixed at 0.10 mL/min, the crossflow field began with an initial crossflow rate of 2.0 mL/min for 3 min, higher than that of Fig. 1. The crossflow rate decreased to 0.5 mL/min over 5 min, then to 0.1 mL/min over 7 min, then to 0.02 mL/min over 5 min and was maintained at 0.02 mL/min until the end of run. With the increase of the initial field strength, a highly degraded NaHA sample containing smaller MW polymer can be well resolved by FI-AFlFFF. The DRI signals of the control sample are shown with open circles of the bottom fractograms in Fig. 3; these showed a broad elution of NaHA material. After an exposure to gamma radiation with a dose of 2 kGy, the DRI fractogram of the 2 kGy sample (filled circles)

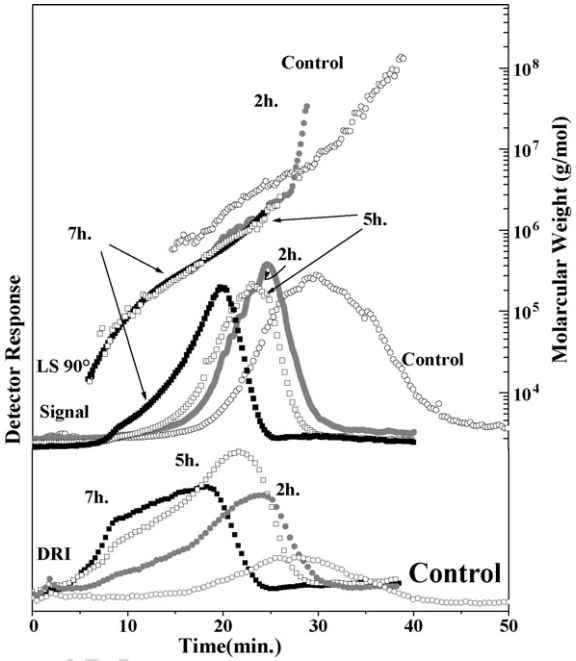

Fig. 3. FlFFF fractograms (DRI signals at the bottom and MALS at 90°) of a raw NaHA sample superimposed with those of the samples degraded by exposure to gamma radiation. MW values corresponding to each sample are plotted with different symbols but the same symbol convention is used. Samples marked with 2, 5 and 7 kGy represent doses of gamma radiation.

provides a significant change in the elution time and the peak shape (narrower) compared to those of the control sample. The peak shifted further to even shorter retention time for the sample exposed to 7 kGy, but the concentration of NaHA molecules eluted at 10 min appeared to increase. When the LS-90° signals shown in the middle of Fig. 3 were examined together with DRI signals, the control NaHA sample (open circles) exhibited a broader distribution from 20 min to ~55 min, which represented higher signal intensities for the late-eluting NaHA molecules. The shift pattern of the LS signals in accordance with the exposure time appeared to be similar to that observed with DRI signals, however, the LS signal intensity of the 7 kGy sample at or around 10 min is not as strong as DRI. The calculated MW values of the control sample (open circles) shown in the upper part of Fig. 3 illustrate that the size fractionation of ultrahigh MW NaHA (up to 10^8 g/mol) was achieved with an increasing order of MW without an abrupt change. From MW values of the 2 kGy sample (filled circles), it is apparent that the majority of NaHA molecules were smaller than $\sim 3 \times 10^6$ g/mol (relatively few data points after this limit). The sharp increase in the MW values for the 2 kGy sample after 3×10^6 is thought to be from erroneous calculation often found at the end of elution where the concentration decreases. For the case of the 7 kGy sample, the lower limit of MW values appeared to be less than 1×10^5 g/mol. Simultaneously, the upper limit of MW values was less than $\sim 1 \times 10^6$ g/mol. From the MW values calculated from MALS/DRI, the decrease of retention of NaHAs upon exposure to gamma radiation provides evidence that the NaHA control sample was degraded into smaller MW polymers. The resulting MWD of each sample is superimposed in Fig. 4a

Table 2
Effect of radiation dose, resulting in degradation, on MW, RMS radius and RSD values ($n=4$) of NaHA samples

	Control sample	Degraded NaHA upon radiation doses		
		2 kGy	5 kGy	7 kGy
Mw (g/mol)	$(8.6 \pm 0.1) \times 10^6$	$(1.5 \pm 0.02) \times 10^6$	$(6.5 \pm 0.04) \times 10^5$	$(4.7 \pm 0.03) \times 10^5$
Mn (g/mol)	$(3.6 \pm 0.03) \times 10^6$	$(9.6 \pm 0.02) \times 10^5$	$(4.5 \pm 0.03) \times 10^5$	$(3.2 \pm 0.02) \times 10^5$
Mw/Mn	2.39 ± 0.04	1.19 ± 0.02	1.45 ± 0.02	1.32 ± 0.01
RMS radius (nm)	118.4 ± 0.5	62.0 ± 0.7	32.5 ± 1.2	31.8 ± 0.2

Radiation listed is the total amount.

which provides a good basis for comparing size distribution. It was found that the ultra large NaHA molecules can be efficiently degraded by gamma radiation from a raw material having weight-average MW of 8.6 ($\pm$0.1) $\times 10^6$ to a product material having a weight-average MW of 4.7 ($\pm$0.03) $\times 10^5$ ($n=4$) after an exposure of 7 kGy. The calculated Mw, Mn and polydispersity values for a variety of exposure doses are compared in Table 2. As shown in Table 2, after being exposed to 7 kGy, the average root-mean-square (RMS) radius value significantly decreased from 118.4 ± 0.5 to 31.8 ± 0.2 nm and the polydispersity (Mw/Mn) was reduced from 2.39 ± 0.04 to 1.32 ± 0.01 ($n=4$).

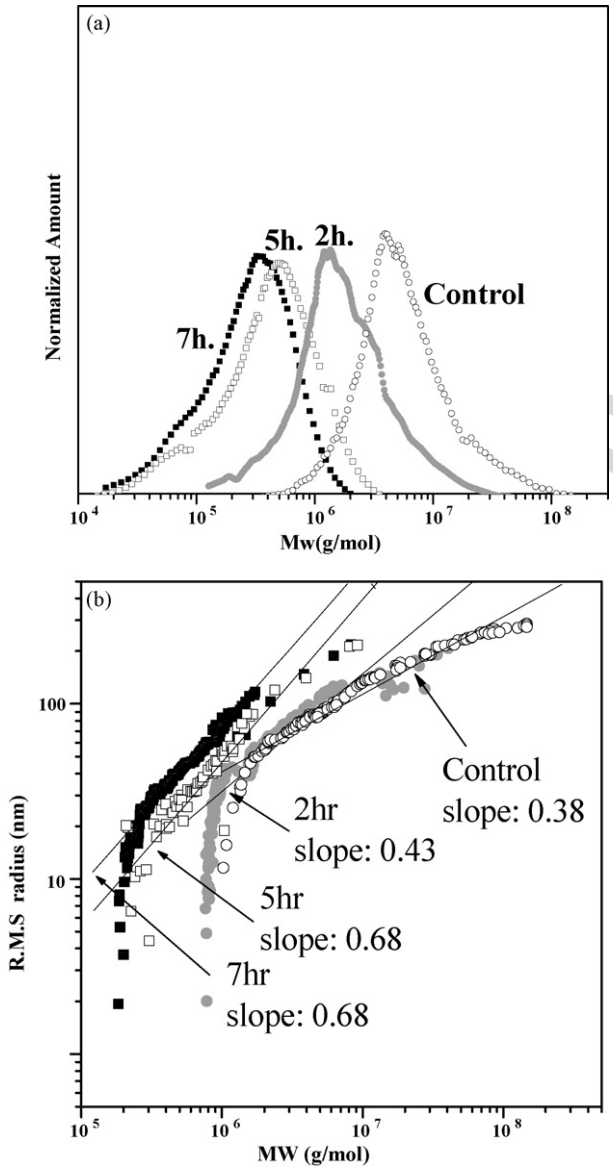

Fig. 4. (a) Area normalized MWD curves of a raw NaHA and three degraded products and (b) plots of RMS radius vs. MW.

Moreover, the MW data points of the degraded NaHA 2 kGy sample in Fig. 3 appeared at a lower position than those of the control sample. The shift of MW data points to a lower region indicates that the retention time of the same molecular weight species decreased with the influence of gamma radiation. This phenomenon can be explained if the conformation of degraded NaHA molecules changed into an unfolded structure, since a linear chain polymer in FlFFF has a shorter retention than a spherical one. The evidence of chain extension of NaHA polymers by gamma radiation can be found from the experimental slope calculation of the plots of RMS radius versus MW in Fig. 4b. The slope value, d(log) RMS versus d(log) MW, of the control sample was calculated to be 0.38, which is expected to correspond to a rather compact structure. The slope increased to 0.43 with 2 kGy of radiation exposure, and further to 0.68 (after 5 kGy of exposure), values known to correspond to a linear shape. From these results, gamma radiation exposure effectively degraded NaHA polymers into smaller MW components and simultaneously altered the conformation of NaHA molecules into an extended linear chain structure.

4. Conclusions

This study demonstrated the utility of FlFFF/MALS/DRI for size fractionation and characterization of ultrahigh MW raw NaHA and their products obtained by refinement processes or gamma radiation degradation. On-line monitoring of eluted NaHA polymers with multiangle light scattering and resulting calculations provided a good comparison of MWDs. This work also showed that a simple refinement process provided different fractions of NaHAs (in terms of MW and viscosity) without changing the conformation. Gamma radiation degradation, however, induced a significant change in MWD and in conformation of NaHA structures.

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