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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

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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

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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.