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Thermal degradation of high molar mass hyaluronan in solution and in powder; comparison with BSA

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ABSTRACT

The aim of the present work was to compare the thermal degradation of bovine serum albumin and hyaluronic acid of different molar masses by determining the loss in molecular weight by means of SEC-MALLS (size exclusion chromatography – multi angle laser light scattering). For all measured samples, the results obtained by this method were compared with the results for stability determined by electrophoretic light scattering. The degradation study was performed in solution and in powder.

Bovine serum albumin (also known as BSA) is a protein derived from cows, which has many biochemical applications. Hyaluronic acid (hyaluronan or HA) is an anionic nonsulfated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues.

The powder and solutions of BSA and HA were heated at different temperatures ranging from 37 °C to 120 °C for certain periods (the highest temperature was used only for the powder). The observed degradation increased with the duration of heating and with temperature for all hyaluronic acid samples analyzed in this work, in accord with Arrhenius law.

At 37 °C and 60 °C, only moderate degradation was observed for hyaluronic acid solutions. For BSA at 37 °C no degradation was observed and at 60 °C significant aggregation occurred.

1. Introduction

Hyaluronic acid, in the form of a sodium salt (hyaluronan or HA) is one of the most important polysaccharides originating in the mammalian body. During the last few decades, many studies have focused on its role in tissues, body fluids, and cell proliferation, and developed ways to use this polysaccharide in wound healing, drug delivery systems, and anti-aging applications. Hyaluronan is a linear natural polysaccharide of the glycosaminoglycans family. Its chemical structure comprises disaccharide units composed of D-glucuronic acid and N-acetyl-D-glucosamine, which are alternatively linked through 1,3 and 1,4 glycosidic bonds [1]. Hyaluronic acid can be synthesized via polymerization reactions [2], as in the case of other polymers obtained by different synthetic processes (co-polymerization, polymerization in solution, enzymatic polymerization etc) [3–6].

Hyaluronan of different molar masses has different roles in the body. For example, high molar mass hyaluronan organizes the extracellular matrix and low molar mass hyaluronan can be found in injured tissue or in certain tumors [7,8]. The stability of hyaluronan and its molar mass are important not only from the point of view of its physiological functions but also in the development of its applications (e.g. tailoring its molar mass to a specific application) and in how it is handled, both in its solid and dissolved form. Many methods of how to cleave hyaluronan were summarized in the review by Stern et al. [9].

Low or high pH conditions have obvious effects on hyaluronan. In acid solution, hydrolysis occurs on the glucuronic acid residue and the hemiacetal ring remains. In basic solution, hydrolysis occurs on the N-acetylglucosamine residue. Such hydrolysis obeys first order kinetics [10]. Other observations suggest that random chain scission occurs during hydrolytic degradation [1,10,11], and this same mechanism has also been proposed for the thermal degradation of hyaluronan [12–14]. The degradation of hyaluronic acid powder using different methods (electron beam irradiation, gamma ray irradiation, microwave irradiation, and thermal treatment) was reported in a study by Choi et al. [15]. The thermal degradation of hyaluronan has not been investigated from the point of view of the decrease in its molar mass. This, therefore, was the main focus of this work, in which the thermal behavior of

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hyaluronan was compared with that of bovine serum albumin, another important biopolymer, well known for its denaturation at elevated temperatures.

Bovine serum albumin (also known as BSA) is a serum albumin protein derived from cows. It is often used as a protein concentration standard in lab experiments (also as Mw standard, for SEC-MALLS normalization). The full-length BSA precursor protein has 607 amino acids (in its length), while the mature BSA protein contains 583 amino acids. Bovine serum albumin (BSA), one of the major components in plasma protein, plays an important role in transporting and metabolizing many endogenous and exogenous compounds in metabolism [15]. Bovine serum albumin can also be synthesized via condensation followed by polymerization reactions, as described in Ref. [16].

We are the first to report a comparative study of the thermal degradation of hyaluronic acid and BSA, in aqueous solution and in solid powder form, using SEC-MALLS. As for BSA in solution, degradation could not be studied at temperatures above 60 °C, because of the significant aggregation which occurred.

In the work published in a study of Yohannes et al. [17], the authors showed that the particle size is relatively stable up to 60 °C, but that above 63 °C aggregation occurs (growth of the hydrodynamic diameter). The heat-induced aggregation of BSA is dependent on temperature, concentration, incubation time, and salt concentration. All these parameters affect the particle sizes of the aggregates, the molar masses, the zeta potential, and the conformational structure of BSA.

For example, the smallest BSA concentration at which aggregates were observed at 80 °C was 0.1 mg/mL[17]. Above this concentration, the sizes of the aggregates were proportionally dependent on BSA concentration. The smallest time for 1 mg/mL BSA solution to aggregate at 80 °C was about 2 min [17].

2. Materials

The hyaluronan samples (sodium salt) used in the present work were purchased from Contipro (Czech Republic). These samples were products of bacterial fermentation (Streptococcus equi., subsp. Zooepidemicus bacterial strain):

- HA 1.67 MDa (1669 kDa): $M_w = 1145.3$ kDa
- HA 1.8 MDa (1800 kDa): $M_w = 1351.5$ kDa

The hyaluronan samples of 1669 and 1800 kDa were 1 year old and 3 years old, respectively. The hyaluronan samples were kept in a refrigerator at 4 °C before use.

The BSA samples used in the present study were purchased from Sigma Aldrich:

- BSA 82 kDa: $M_w = 82.3$ kDa;
- BSA 87 kDa: $M_w = 87$ kDa.

The used BSA samples of 82 and 87 kDa were 2 years old and 4 years old, respectively, and were also kept in the refrigerator at 4 °C prior to analysis.

3. Methods

The principal technique used to study thermal degradation in this study was SEC-MALLS, which gives detailed information on polymer molar mass and conformation.

The SEC-MALLS apparatus used in the present work was produced by Wyatt (USA; the detector part) and by Agilent (USA; the chromatography part). It included a MALLS detector (Dawn Heleos II), a viscometric detector (ViscoStar II), and a refractive index detector (Optilab T-rEX). The multi angle laser light scattering (MALLS) detector had 18 angles of detection, ranging from 10° to 160°. The Astra 6 software package was used for data collection and analysis.

The chromatograph contained a degasser, an isocratic pump, an autosampler, a column for size exclusion chromatography (one PL aquagel-OH MIXED-H 8 μm PL1149-6800 produced by Agilent), and a thermostat. The used mobile phase was 0.1 M $NaNO_3$ aqueous solution, containing 3 mM NaN_3 to prevent the growth of microorganisms. All SEC-MALLS measurements were performed at 25 °C.

The solutions were prepared using the same solvent as that used for the mobile phase. Concentrations of 1 mg/mL for hyaluronan and 5 mg/mL for BSA were used. All samples were filtered before injection using Millipore Durapore Membrane filters 0.1 μm.

The SEC-MALLS technique allows:

- the separation of different polymeric compounds (fractions) according to their molar masses
- the determination of absolute molar mass averages from 10^2 Da to 10^9 Da
- the calculation of polydispersity
- the determination of the root mean square radius (RMS), also known as the “radius of gyration” (R_g)
- the determination of the conformation plot and Mark–Houwink–Sakurada (MHS) plot

From the slope of the conformation plot RMS $= f(M_w)$, the shape of the polymer can be found [18–20]. The shape of the polymer can also be obtained from the Mark–Houwink–Sakurada plot [18,21–24].

For molar mass calculation, the Zimm model was used [25,26]. The refractive index increment (dn/dc) values used to calculate the molar mass were 0.185 mL/g for BSA and 0.165 mL/g for hyaluronic acid [27,28].

The results obtained from SEC-MALLS were supplemented with the charge characterization of the colloidal stability of dissolved samples, as measured by the method of electrophoretic light scattering (ELS) on a Zetasizer Nano ZS (Malvern Instruments). The ELS method measures the movement of charged colloidal particles in the sample after the application of an electric field. First, the electrophoretic mobility of particles in the sample is determined and this value is recalculated to zeta potential using the Henry equation [29]. The value of the zeta potential is used to estimate the stability of the sample resisting aggregation (the colloidal stability due to the electrostatic repulsion). If the value of the zeta potential is between −30 and 30 mV, the charge of the particles is not sufficient to ensure the stabilization of repulsive forces; that is, particles can approach each other closely and aggregation occurs. In this case, the sample is non-stable. If the zeta potential is higher than 30 mV or lower than −30 mV, particles have sufficient surface charge and these systems are considered to be stable against aggregation.

Electrophoretic light scattering measurements for all studied samples were performed using similar settings as in the SEC-MALLS method (temperatures of 37, 60, or 90 °C in solution; total time of heating ranging from 1 to 12 h). All measurements were conducted four times and the obtained results are presented as mean value ± standard deviation.

4. Results and discussion

We analyzed two hyaluronan samples of different molar mass and two BSA samples of different molar mass after their thermal degradation in powder form and in solution. Thermal treatment of the analyzed samples was performed at different temperatures for certain periods.