Evaluation of Radius of Gyration and Intrinsic Viscosity Molar Mass Dependence and Stiffness of Hyaluronan

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Nine hyaluronan (HA) samples were fractionated by size-exclusion chromatography, and molar mass (M), radius of gyration (Rg), and intrinsic viscosity ([η]) were measured in 0.15 M NaCl at 37 °C by on-line multiangle light scattering and viscometer detectors. Using such method, we investigated the Rg and [η] molar mass dependence for HA over a wide range of molar masses: M ranging from 4 × 10^4 to 5.5 × 10^6 g/mol. The Rg and the [η] molar mass dependence found for HA showed a meaningful difference. The Rg = f(M) power law was substantially linear in the whole range of molar masses explored with a constant slope of 0.6. In contrast, the [η] = f(M) power law (Mark–Houwink–Sakurada plot) showed a marked curve shape, and a linear regression over the whole range of molar masses does not make sense. Also the persistence length (stiffness) for HA was estimated. The persistence length derived by using both the Odijk’s model (7.5 nm from Rg vs M data) and the Bohdanecky’s plot (6.8 nm from [η] vs M data) were quite similar. These persistence length values are congruent with a semistiff conformation of HA macromolecules.

Introduction

A fundamental aspect in understanding the physical properties of a polymer involves determination of the dimension of the macromolecules in solution as a function of the molar mass. In such context, an accurate determination of the root-mean-square radius, hereafter denoted in short as radius of gyration (Rg), vs molar mass power law and of the intrinsic viscosity ([η]) vs molar mass power law, generally known as Mark–Houwink–Sakurada (MHS) plot, are of fundamental importance. Theory of the Rg = f(M) and [η] = f(M) scaling functions assumes homogeneous monodisperse fractions in molar mass and dimension of the macromolecules. Hence, a preliminary step in such a study is the fractionation of the starting polydisperse polymer. Off-line fractionation of broad molar mass distribution (MMD) polymers is time-consuming and tedious, and more important, the final results are often not adequate, that is, the MMD of the final fractions is again relatively broad. Alternatively, the Rg = f(M) and [η] = f(M) power laws are determined using broad MMD polymers. Eventually, some authors used theoretical corrections for the polydispersity of the samples.1

In general, however, the influence of the polydispersity of the samples on the final results is meaningful, and the validity of the theoretical corrections is often doubtful. Consequently, an accurate fractionation of the broad MMD samples should produce more accurate results.

The on-line fractionation of broad MMD samples by size-exclusion chromatography (SEC) is rapid and efficient and requires a small amount of the polymer. Recently, the use of molar mass and viscosity sensitive detectors coupled on-line to a SEC system has become relatively common in many laboratories.2 Virtually, a multiangle laser light scattering (MALS) photometer, along with a viscometer, provides directly M, Rg, and [η], of each eluting fraction. Assuming ideal SEC fractionation, each fraction eluted from the chromatographic column could be considered homogeneous (monomolecular) in molar mass, and at least in principle, the Rg = f(M) and [η] = f(M) power laws can be obtained from one single broad MMD sample during one single SEC run.

Hyaluronan, which is the sodium salt of hyaluronic acid, is a linear biopolymer. HA is a regularly alternated polysaccharide composed of N-acetylglucosamine and D-glucuronic acid. HA, in origin either extractive or bacterial, is water-soluble, and in aqueous solutions, it is a negatively charged polyelectrolyte. The MMD of the native HA samples is relatively broad; the molar mass ranges from medium to high up to ultrahigh. There is large industrial interest for HA as attested by the extensive use in medicine, cosmetics, etc.3 Furthermore, there is a large scientific interest for the HA properties in solution. In the past, many authors have investigated Rg and [η] of HA as a function of the molar mass.4–10 Several studies concerned also the HA macromolecule conformation in aqueous solution, and in particular, the stiffness of the macromolecules (persistence length).11–20 Really, the study of Rg and [η] as a function of the molar mass, ionic strength, etc. is a main concern for HA. To the best of our knowledge, almost all of the previous studies on the size and the conformation of HA macromolecules, with very few exceptions,8 used off-line methods and unfractiated samples having broad MMD. In contrast, our goal...
presented here was to study $R_g$ and $[\eta]$ molar mass dependence, together with the persistence length of HA, by exploiting simultaneously the SEC fractioning and MALS and viscosity on-line detecting capabilities.

**Experimental Section**

**Materials.** The study was performed using nine HA samples with the weight-average molar mass, $M_w$, ranging from $9.1 \times 10^4$ to $3.5 \times 10^6$ g/mol (Table 1). Sample sources were the following: HA_01 and HA_07 from Genzyme Corporation (Cambridge, MA); HA_02 and HA_03 from Pentapharm AG (Basel, Switzerland); HA_04 from CPN LTD (Ústí nad Orlicí, Czech Republic); HA_05, HA_08, and HA_09 from Pharmacia & Upjohn (Nerviano, Milan, Italy); HA_06 from Lifecore Biomedical Inc. (Chaska, MN). Each HA sample contained typically less than 0.2% of proteins. Bovine serum albumin (BSA) was obtained from Sigma (Milan, Italy). Water was MilliQ grade (Millipore, Bedford, MA). All other chemicals were of analytical grade.

**Chromatographic System.** The study was performed with a multidetector SEC chromatographic system using three online detectors: a homemade single capillary viscometer (SCV), a MALS Dawn DSP-F photometer from Wyatt (Santa Barbara, CA), and a UV 996 from Waters (Milford, MA) as a concentration detector. This multidetector SEC system was described in detail previously. The experimental conditions consisted of 0.15 M NaCl as mobile phase at 37 °C, 0.2 mL/min flow rate, and 200 µL injection volume. The column set was composed of two TSKgel PW (G6000 and G5000, 17 µm particle size) from Tosohaas (Montgomeryville, PA).

**Light Scattering.** The MALS photometer uses a vertically polarized He–Ne laser ($\lambda = 632.8$ nm) and simultaneously measures the intensity of the scattered light at 15 angular locations ranging in aqueous solvent from 14.5° to 158.3°. The calibration constant was calculated using toluene as standard assuming a Rayleigh factor of 1.406.$^{10}$

The calibration constant was calculated using toluene as standard assuming a Rayleigh factor of 1.406.$^{10}$ The original homemade SCV detector used for viscometry. The three-detector system (MALS–SCV–UV) exploited allows us to determine directly the two investigated relationships, namely, the $R_g = f(M)$ and $[\eta] = f(M)$ power law functions (shown, respectively, in Figures 2 and 3. Both plots, in double logarithmic scale, indicated relatively straight lines. Thus the parameters (intercept, slope) of both lines were calculated. Respectively, $R_g$ was expressed in nanometers and $[\eta]$ in milliliters per gram.

$$R_g = 2.53 \times 10^{-2}(M^{0.601})$$

$$[\eta] = 3.21 \times 10^{-2}(M^{0.783})$$

It should be pointed however here, that the parameters calculated were determined simply by SEC analysis of the sample HA_05 alone. Analogously, by analyzing the all nine HA samples, one can generate nine pairs of relationships analogous to those described above. However, another and more meaningful analysis could be done by gathering together all nine sets of $R_g$ vs $M$ data. As evident from Figure 4, the superposition of all nine individual $R_g$ vs $M$ dependencies resulted in one single relationship valid for all nine analyzed samples. The $R_g = f(M)$ power law function in

**Table 1. Summary of More Relevant Data for Nine HA Samples**

<table>
<thead>
<tr>
<th>sample</th>
<th>$M_w$ (kg/mol)</th>
<th>$D$</th>
<th>$R_g$ (nm)</th>
<th>$[\eta]$ (mL/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA_01</td>
<td>91.0</td>
<td>1.8</td>
<td>28.3</td>
<td>250.1</td>
</tr>
<tr>
<td>HA_02</td>
<td>236.2</td>
<td>2.2</td>
<td>55.0</td>
<td>526.0</td>
</tr>
<tr>
<td>HA_03</td>
<td>580.0</td>
<td>1.8</td>
<td>89.0</td>
<td>1060.9</td>
</tr>
<tr>
<td>HA_04</td>
<td>665.0</td>
<td>2.1</td>
<td>98.0</td>
<td>1173.2</td>
</tr>
<tr>
<td>HA_05</td>
<td>1060</td>
<td>1.8</td>
<td>126.1</td>
<td>1660.4</td>
</tr>
<tr>
<td>HA_06</td>
<td>1210</td>
<td>1.6</td>
<td>120.4</td>
<td>1796.8</td>
</tr>
<tr>
<td>HA_07</td>
<td>1400</td>
<td>1.7</td>
<td>132.9</td>
<td>1962.0</td>
</tr>
<tr>
<td>HA_08</td>
<td>1650</td>
<td>1.8</td>
<td>163.3</td>
<td>2165.1</td>
</tr>
<tr>
<td>HA_09</td>
<td>3500</td>
<td>1.6</td>
<td>257.7</td>
<td>3402.2</td>
</tr>
</tbody>
</table>

Figure 1. UV detector signal of the HA_05 sample and molar mass, radius of gyration, and intrinsic viscosity vs elution volume experimental functions.
Figure 4 can be well described by one single equation:

\[ R_g = 2.75 \times 10^{-2}M^{0.596} \]

The \( R_g = f(M) \) power law dependence estimated is a nice straight line approximately ranging in molar mass between 40 and 5500 kg/mol and in radius of gyration between 15 and 290 nm. It should be pointed out here that, as expected, the power law function \( R_g = f(M) \) obtained by analyzing one single sample (HA_05) and that function generated by superpositioning all nine HA samples have a near identical slopes (~0.6) and closely identical intercepts.

Figure 5 represents the results of the analogous procedure performed by superpositioning the nine individual [\( \eta \)] vs \( M \) dependencies obtained by SEC analysis of the nine HA samples. In this plot, [\( \eta \)] ranged approximately from 80 to 4330 mL/g. As evident, the relationship [\( \eta \)] vs \( M \) indicated a marked nonlinearity. Consequently, a linear regression over the whole range of the [\( \eta \)]-\( M \) data is evidently not acceptable. However, only for comparison with the MHS linear coefficients for HA published previously in the literature the whole range of the [\( \eta \)]-\( M \) data was divided into three subregions, namely, (a) \( M < 1 \times 10^5 \) g/mol; (b) \( 10^5 < M < 10^6 \) g/mol; (c) \( M > 10^6 \) g/mol. By such a way, three particular [\( \eta \)] = \( f(M) \) power law functions were calculated:

\[ [\eta] = 1.29 \times 10^{-3}M^{1.056} \quad \{M < 10^5 \text{ g/mol}\} \]

\[ [\eta] = 3.39 \times 10^{-2}M^{0.778} \quad \{10^5 < M < 10^6 \text{ g/mol}\} \]

\[ [\eta] = 3.95 \times 10^{-1}M^{0.604} \quad \{M > 10^6 \text{ g/mol}\} \]

Figure 5 shows the previous three MHS equations for HA. Figure 6 is just another presentation of the procedure described above. As evident, the two individual HA samples, namely, HA_01 and HA_09, that is, those having the lowest and the highest molar mass, resulted in two particular, not identical, [\( \eta \)] = \( f(M) \) power law functions. However, the power law functions \( R_g = f(M) \) for these two samples are identical (not shown).

**Discussion**

The on-line SEC–MALS–SCV system is a powerful tool to estimate the molecular parameters of the HA macromol-
molecules. Really, this system allows the estimation of the $R_g = f(M)$ and $[\eta] = f(M)$ power laws from homogeneous or at least very narrow HA fractions. Obviously by “fraction”, we mean each slice eluted from the SEC column. The scaling functions, $R_g = f(M)$ and $[\eta] = f(M)$, are very important because they furnish relevant information on the stiffness of the macromolecules. HA stiffness in aqueous solution has been studied in the course of the years by many authors. In general, the hypothesis of semistiff conformation for HA found large consensus. Many MHS linear coefficients could be found in the literature,\textsuperscript{7,10} as well as $R_g = f(M)$ coefficients and persistence length values.\textsuperscript{11-20} Although not all of the published values for HA seem to converge, the previous assertion on the HA stiffness is accepted from the majority of authors. Usually $R_g = f(M)$ and MHS coefficients and persistence length values for HA have been estimated using several HA samples with different molar mass. Unfortunately, all of the HA samples present a relatively broad MMD. HA polydispersity influences to some extent the recovered value of the coefficients of the power laws and of the persistence length. Hence, our results obtained after SEC fractionation over a very wide range of molar mass is particularly meaningful.

$R_g = f(M)$ Power Law. The slope of the $R_g = f(M)$ power law calculated with a linear regression over the whole set of $R_g-M$ data of the nine HA samples was 0.6. More in detail, the slopes of the individual $R_g = f(M)$ power laws of the nine HA samples were quasi-constant ranging from 0.592 to 0.604. The experimental data of the $R_g = f(M)$ plot approximately cover a $R_g$ range from 15 to 290 nm. In this explored range, the $R_g = f(M)$ power law was substantially linear. However, the theoretical $R_g = f(M)$ power law calculated, for example, with the Odijk’s model, described in detail in the following text, predicts a curvature of the plot in the lower $R_g$ range, but our experimental data does not cover this low $R_g$ interval. Our finding on the slope of the $R_g = f(M)$ power law is a little higher than the value (0.57) obtained from Fouissac\textsuperscript{14} in quasi-similar conditions (0.1 M NH$_4$NO$_3$). In addition, in a previous study\textsuperscript{18} using unfractonated HA samples with broad MMD, we found a lower slope value (0.57). Evidently the polydispersity of the broad HA samples used in those studies thus influenced the recovered slope result.

$[\eta] = f(M)$ Power Law. Very interesting is the marked curvature of the MHS plot for HA. Although the curvature of the MHS plot is not an original result but is the normal behavior of a wormlike chain, the interest arises from the experimental evidence of this quite unusual behavior. We have anticipated in the Results section that we have divided the molar mass range into three subregions only for comparison with MHS coefficients for HA published previously in the literature. Evidently, this procedure is an artifice because the MHS plot for HA is not divided into three linear subregions but shows a continuous curvature. However, many MHS linear coefficients were published for HA in the course of the years, and a comparison in the corresponding molar mass range could be interesting. In brief, our findings are (1) $a \approx 0.78$ with $M$ ranging from 100 to 1000 kg/mol, (2) $a \approx 0.6$ with $M$ higher than 1000 kg/mol, and (3) $a \approx 1.06$ with $M$ lower than 100 kg/mol. Different values of the slope of the MHS plot on the basis of different ranges of molar mass is not new for HA. Bothner\textsuperscript{7} provided two sets of MHS coefficients. The first one was valid for $M$ lower than 10$^6$ g/mol ($a = 0.779$); second one was valid for $M$ higher than 10$^6$ g/mol ($a = 0.601$). Hence, also Bothner’s experimental results reflected a different slope of the MHS plot for HA in different ranges of molar mass. Furthermore, other authors\textsuperscript{6,11} reported values of the slope of the MHS plot approximately 1 for HA with $M$ lower than 10$^5$ g/mol. In particular, Cowman and Matsuoka\textsuperscript{9} predict $a = 1.16$ for HA with $M$ lower than 37.5 kg/mol. The nonlinearity of the MHS plot reflects the conformational change of the HA chain on basis of the chain length. Cleland\textsuperscript{5} explained the conformational change of HA in terms of wormlike chain or non-Gaussian behavior of short chains at lower molar mass and flexible chains or Gaussian behavior of long chains at higher molar mass. Cowman and Matsuoka\textsuperscript{9} assert that short HA chains act like free-draining chains and long HA chains act like non-free-draining coils. These considerations explain quite well the strong changes of the slope of the MHS plot for HA when a wide range of molar masses is explored.

Considering the coefficients of the central part of the MHS plot, 100 < $M$ < 1000 kg/mol, which are more meaningful from a practical point of view, we point out that they are in agreement with part of the published values for HA in similar conditions. Many MHS coefficients for HA have been published in the course of the years. Unfortunately, part of the published MHS coefficients disagree. Several differences could explain these discrepancies: (i) origin of the HA samples (extractive from varied source or fermentative); (ii) experimental conditions (shear rate, ionic strength, pH, temperature); (iii) molar mass range. In addition, $[\eta]$ measure is relatively complex in consequence of the non-Newtonian behavior of HA dilute solutions when the molar mass is high. However, despite the relative meaning of the linear analysis of the $[\eta]$ vs $M$ data, we point out that our MHS coefficients are in quite good agreement with several previously published results.\textsuperscript{4,7,8} Eventually, there is only a little discrepancy in the intercept $k$ that could be explained with the higher temperature 37 °C used in our study against 25 °C of the Bothner study.\textsuperscript{7}

An interesting point arises from the difference between $R_g$ and $[\eta]$ molar dependence. Really, it is not immediate to understand why only the $[\eta]$ power law results in a nonlinear dependence. It is well-known that the $R_g$ data are the results of equilibrium measurements while for $[\eta]$ data this is not the case. In addition, the “partial draining” nature of charged chains changes with their length, that is, their hydrodynamic volume changes with $M$. As a consequence, it is interesting to note that the so-called “universal constant” $\Phi$, which relates $[\eta]$ and the dimension of the macromolecule, is not a constant.

Persistence Length. Using the molar mass dependence of the radius of gyration and of the intrinsic viscosity, one can estimate the stiffness of the HA chain via the persistence length, $q$. In the literature, $q$ values recovered from $R_g$ data or in alternative from $[\eta]$ data do not agree. Several different $q$ values ranging from 2 to 20 nm were published.\textsuperscript{11,15} Cleland\textsuperscript{5,11} found $q = 4-5$ nm for HA in aqueous NaCl. A quite similar value ($q = 4.1-4.2$ nm) was found from
contributions: the electrostatic persistence length, \( q_e \), and the electrostatic expansion factor, \( \alpha_{S,El} \).

The electrostatic persistence length, \( q_e \), substantially depends on the ionic strength of the solvent. A method to calculate \( q_e \) for a specific ionic strength is reported in the Fouissac article.\(^{14}\) Specifically, \( q_e \) for HA in 0.15 M NaCl is relatively low, 0.11 nm. For \( \alpha_{S,El} \), Odijk adopted the Yamakawa–Tanaka\(^{26}\) approximation, and the electrostatic expansion factor is given by

\[
\alpha_{S,El} = 0.541 + 0.549(1 + 6.044q_e^{0.46})
\]

where \( q_e \) is the electrostatic excluded volume parameter, \( \beta_{El} \) is the electrostatic excluded volume between two rods of length \( \lambda^{-1} \), and \( \kappa^{-1} \) is the Debye radius.

Using eqs 1–5, assuming\(^{15}\) \( M_L = 410 \text{ nm}^{-1} \), we calculated the theoretical \( R_g \) molar mass dependence. The best superimposition between the experimental and theoretical \( R_g \) vs \( M \) data was obtained with an intrinsic persistence length \( q_0 \) of 7.5 nm. Figure 7 shows the comparison of the experimental and theoretical \( R_g \) molar mass dependence for HA in 0.15 M NaCl calculated with the Odijk’s model and \( q_0 = 7.5 \text{ nm} \).

Alternatively, the persistence length \( q \) may be derived using the molar mass dependence of the intrinsic viscosity and the Bohdanecy’s procedure.\(^{28}\) Bohdanecy showed that the Yamakawa–Fuji theory\(^{26}\) for wormlike macromolecules can be approximated with the following equation:

\[
\left( \frac{M^2}{[\eta]_0} \right)^{1/3} = A_q + B_q M^{1/2}
\]

\( A_q \) and \( B_q \) parameters can be easily estimated by a linear regression over the whole set of experimental data. \( A_q \) and \( B_q \) parameters are related to \( M_L \) and \( \lambda^{-1} \) by the following equations:

\[
A_q = \Phi_{0,w}^{-1/3} A_{0,w} M_L
\]

\[
B_q = \Phi_{0,w}^{-1/6} B_{0,w} \left( \frac{1}{M_L} \right)^{1/2}
\]

where \( \Phi_{0,w} = 2.86 \times 10^{-23} \text{ mol}^{-1} \) is the limiting value of the Flory’s constant \( \Phi \) for nondraining coils in the limit of infinite molar mass. Both \( A_{0,w} \) and \( B_{0,w} \) are known functions of the reduced hydrodynamic diameter, \( d_e = d\lambda^{-1} \), and are tabulated in the Bohdanecy’s article.\(^{28}\) In essence, \([\eta]\) depends on three parameters: \( q \), \( M_L \), and the hydrodynamic diameter, \( d \). In the estimation of these three parameters, we need an additional relation. Bohdanecy proposed the use of the following equation:

\[
d = \left( 4\pi M_L / (\pi N_A) \right)^{1/2}
\]

where \( v \) is partial specific volume at infinite dilution (\( v = 0.57 \text{ cm}^3 \text{g}^{-1} \) for HA in NaCl salt\(^{29}\)) and \( N_A \) is the Avogadro’s
number. Furthermore, in calculating the unperturbed \([\eta]_0\) values, one needs the expansion factor for the intrinsic viscosity, \(\alpha_\eta\). The expansion factor for the intrinsic viscosity \(\alpha_\eta\) is defined in eq 10 and is a complex function of the expansion factor for the radius of gyration, \(\alpha_S\). Weill and Cloizeaux derived a semiempirical relation, eq 11, between \(\alpha_\eta\) and \(\alpha_S\).

\[
[\eta] = [\eta]_0 \alpha_\eta^3
\]

\[
\alpha_\eta^3 = \alpha_S^{2.43}
\]

Figure 8 shows the Bohdanecky’s plot of our experimental data. Very interestingly, the Bohdanecky’s plot is linear over the whole set of HA experimental data, and the estimation of the intercept \(A_\eta\) and of the slope \(B_\eta\) was relatively simple. Using the \(A_\eta\) and \(B_\eta\) values and the eqs 6–11, we have obtained the following results: \(q = 6.8\) nm, \(d = 0.8\) nm, and \(M_L = 480\) nm\(^{-1}\). We point out that a hydrodynamic diameter \(d = 0.8–1\) nm is a well-accepted value for HA. On the contrary, \(M_L = 480\) nm\(^{-1}\) is a little bit higher of the accepted value (400–410 nm\(^{-1}\)). On the whole, the Bohdanecky’s procedure applied to our \([\eta]–M\) experimental data has furnished congruent results. In addition, the agreement between the persistence length value recovered with the Odijk’s model (7.5 nm from \(R_g\)), and the Bohdanecky procedure (6.8 nm from \([\eta]\)) is quite good considering that the evaluation of the stiffness of a macromolecular chain through the persistence length is a very delicate task.

Conclusions

We have found that the \(R_g = f(M)\) power law for HA is linear over the whole range of molar masses explored with a quite constant slope value of 0.6. In contrast, the \([\eta] = f(M)\) power law for HA shows a marked curvature, and a single linear regression over the whole range of molar masses explored does not make sense. It is well-known that such curvature is the normal behavior of wormlike chains. Only for comparison with previous published MHS coefficients for HA, we have also estimated three different sets of coefficients of the MHS plot. The slope of the MHS plot was approximately 1.06 for \(M\) lower than 100 kg/mol, 0.78 for \(M\) ranging from 100 to 1000 kg/mol, and 0.6 for \(M\) higher than 1000 kg/mol. We have also found that the persistence length for HA was 7.5 nm using the Odijk’s model and the \(R_g\) vs \(M\) data and 6.8 nm using the Bohdanecky’s plot and the \([\eta]\) vs \(M\) data. We consider our findings particularly meaningful because they were obtained using SEC fractionated HA samples (theoretically monodisperse fractions) and absolute, MALS, and SCV on-line detectors. Our results are congruent with a semistiff conformation for HA.

References and Notes

16. Reed, W. Macro-ion characterization from dilute solutions to complex fluids; ACS Symposium Series 548; American Chemical Society: Washington, DC, 1994; p 315.