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Fractionation and characterization of ultra-high molar mass hyaluronan: 2. On-line size exclusion chromatography methods

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Abstract

Present study concerns SEC fractionation of ultra-high molar mass hyaluronan. Problems in SEC of UHMM HA samples are the fractionation in the columns and the calibration of the system. We have overcome the calibration problem using absolute detectors, light scattering and viscometer, on-line to the SEC system that do not need of calibration. Shear degradation, concentration effects, viscous fingering, poor column resolution, and in general, low reproducibility are the main difficulties in SEC fractionation. A successful characterization of UHMM HA polymers requires an optimization of the experimental protocol. Each step of the experimental protocol should be performed methodically to obtain reliable results. Commercially available SEC aqueous columns have not optimized for UHMM polymers. Using an optimized SEC system shear degradation and non-ideal SEC fractionation can occur when M_w of the HA sample is approximately higher than three million. Also the dispersity index could be underestimated. © 2002 Published by Elsevier Science Ltd.

Keywords: Hyaluronan; Ultra-high molar mass; Size exclusion chromatography

1. Introduction

Hyaluronan (HA) or hyaluronate, i.e. the sodium-salt of hyaluronic acid, is an unbranched regularly alternated disaccharide composed of *N*-acetylglucosammine and D-glucuronic acid. HA origins could be extractive, from varied source, and bacterial. HA is water-soluble and in solution is a negatively charged polyelectrolyte. Generally HA molar mass distribution (MMD) is relatively broad and the molar mass ranges from high to ultra-high (it has been reported up to 1×10^7 g/mol). There is large industrial and scientific interest in regard to HA, particularly for ultra-high molar mass (UHMM) HA. The industrial interest has attested from the extensive use of HA in medicine, biosurgery, etc. The scientific interest has been attested by the number of publications that have been issued over the last sixty years [1–10]. A more complete list of the literature regarding HA characterization could be found in the overview published by Chabrecek et al. [11]. Despite this large interest relevant and congruent data do not exist on the fractionation by size exclusion chromatography (SEC) technique of UHMM HA samples.

The complex biological functions of HA are closely related to the viscoelastic properties and consequently to the MMD. Various off-line methods such as light scattering, viscometry, osmometry and sedimentation could be used for the molecular characterization of UHMM HA. In a previous paper [12] we have presented the characterization by off-line light scattering and viscometry of UHMM HA with the weight-average molar mass M_w up to 1×10^7 g/mol. However, many HA specific functions depend on the whole MMD rather than an average molar mass value. Hence, we were interested to the fractionation and the characterization of the whole MMD. Different methods could be used for the on-line fractionation of UHMM HA. Virtually, there are three alternatives: SEC, hydrodynamic chromatography and field flow fractionation (FFF). For our goal probably a fractionation by means of a flow-FFF system [13] could be more effective. However, SEC is by far the most common method of fractionation, consequently, there is interest to explore the extreme potentialities of this technique in the fractionation of UHMM HA. Hence, this study will face only the fractionation of UHMM HA by SEC columns.

It is well known that HA macromolecules in aqueous solvent are extraordinarily swollen and assume semi-stiff conformation. Consequently, the dimensions of UHMM HA

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macromolecules are very large [12]. The dimensions of UHMM HA macromolecules lie in the borderline between macromolecules and particles. Every time SEC fractionation was applied to UHMM macromolecules or particles severe problems were invariably reported [14–17]. In effect the fractionation of UHMM polymers by SEC is very difficult. Main problems in the SEC characterization of UHMM samples are the fractionation in the columns and the calibration of the system. We have overcome the calibration problem using absolute detectors, light scattering and viscometer, on-line to the SEC system that do not need of calibration. Substantially, this study is an overview of performances and problems of some commercially available aqueous SEC columns in the fractionation of UHMM HA.

2. Experimental

2.1. Materials

This study was performed using several HA samples with M_w ranging from high to ultra-high: from 2.3×10^5 to 7.4×10^6 g/mol. Some HA samples, M_w from 1.06×10^6 to 7.4×10^6 g/mol, both of extractive (rooster comb) and fermentative source were obtained from Pharmacia and Upjohn (Nerviano, Milan, Italy). Three HA samples ($M_w = 4.3 \times 10^5$, 6.6×10^5 and 1.4×10^6 g/mol) were kindly supplied by Dr Ladislav Soltes of the Institute of Experimental Pharmacology of the Slovak Academy of Sciences. Lower molar mass HA sample ($M_w = 2.3 \times 10^5$ g/mol) was of extractive (rooster comb) source. All HA samples were highly purified, typically contained less than 0.2% of proteins. Bovine serum albumin (BSA) was obtained from Sigma (St Louis, MO, USA). Water solvent was MilliQ grade from Millipore (Bedford, MA, USA). All other chemicals were of analytical grade.

2.2. Chromatographic system

An original multidetector SEC chromatographic system, obtained by assembling three different instruments, has been used. The system consisted of a 150CV system from Waters (Milford, MA, USA) with a homemade single capillary viscometer (SCV), a concentration detector and an additional multi-angle laser light scattering (MALS) Dawn DSP-F from Wyatt (Santa Barbara, CA, USA). As concentration detector we have used both an UV (996 from Waters) and a differential refractometer (DRI, 410 from Waters). The set-up of the detectors was serial in the following order: SCV–UV–MALS–DRI. The MALS detector was located after the UV detector because its cell volume is relatively large. In such a way the UV detector, on the contrary to DRI, was not affected from the local band broadening in the MALS cell. This multi-detector SEC system has been described in detail previously [18]. The experimental conditions consisted of 0.15 or 0.5 M NaCl as mobile phase, 37 °C of temperature, 0.2 ml/min of flow rate and 200 μl of injection volume. We have tested four aqueous column sets: (1) 2 TSKGel, G6000PW and G5000PW, from TosoHaas (Stuttgart, Germany); (2) 2 OHpak Shodex, KB-806 and KB-805, from Showa Denko (Tokyo, Japan); (3) 2 PL aquagel-OH 60 15 μm from Polymer Laboratories (Shroshire, UK); (4) 2 Ultrahydrogel columns, 2000 and 1000 Å, from Waters.

2.3. Light scattering

The MALS photometer uses a vertically polarized He–Ne laser of 632.8 nm of wavelength and simultaneously measures the intensity of the scattered light at 16 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^{-5} cm^{-1}. The photodiodes normalization was performed by measuring the scattering intensity in the solvent of a BSA globular protein assumed to act as an isotropic scatterer. Details of the MALS detector have been described elsewhere [19] and will not reported herein. It is well known that the MALS on-line detector measures, at each elution volume V, the molar mass M and the dimension of the macromolecules in the following denoted in short as gyration radius R_g. The refractive index increment, dn/dc, for HA was assumed as 0.150 ml/g [12].

2.4. Viscometer

The on-line viscometer was a homemade SCV. Details of this on-line detector have been described elsewhere [18]. The dimensions of the capillary tube were 0.02 in. of internal diameter and 20 in. of length. The signal of the on-line viscometer depends on the intrinsic viscosity $[\eta]$ and on the concentration c of the solution. Hence, to obtain constant signal to noise ratio the concentration of the HA samples was adjusted so that $[\eta]c = 0.1$. The SCV on-line detector measures the intrinsic viscosity at each elution volume $[\eta]_i$.

3. Results and discussion

This article follows the study on the characterization of UHMM HA by static off-line methods [12]. This second part is focused to the fractionation by on-line SEC of the same UHMM HA samples used in the off-line characterization. M_w, R_g and $[\eta]$ averages obtained by off-line methods will be used as reference for the on-line results. In this way, it has been checked the potential degradation of UHMM HA samples in the SEC columns. More relevant data of a selection of HA samples obtained by static off-line methods are summarised in Table 1. Table 1 also reports the concentration of the sample solutions used in the SEC characterization and for comparison also the overlapping concentration ($c^* \approx [\eta]^{-1}$). The c/c^* ratio of each UHMM

Table 1
Summary of more relevant data for HA samples in 0.15 M NaCl at 37 °C

Sample	$M_w \times 10^{-6}$ (g/mol)	R_g (nm)	$[\eta]$ (dl/g)	c (mg/ml)	c^* (mg/ml)
HA_01	0.23	54.8	5.2	0.23	1.93
HA_03	0.66	98.0	11.6	0.10	0.88
HA_04	1.06	126.1	16.5	0.07	0.61
HA_05	1.65	163.3	21.7	0.05	0.43
HA_07	3.50	257.7	34.0	0.04	0.29
HA_09	5.00	300.5	42.2	0.03	0.24
HA_12	7.40	385.0	53.4	0.02	0.18

c: sample concentration; c^* : overlapping concentration.

HA solution was approximately 0.1 confirming the very dilute concentration of the solutions.

3.1. Optimization of the SEC system

Typical SEC experimental conditions applied to the fractionation of UHMM HA present several drawbacks as shear degradation, concentration effects, viscous fingering, and in general, poor resolution. Many aspects of the SEC experimental protocol that are not critical in the usual molar mass range become determining with UHMM polymers. Using an experimental protocol not optimized for UHMM HA the chromatogram is abnormal (bimodal, tailed) and the calibration curve, from on-line MALS, do not assume the usual monotonous decreasing course but a sequence of wave, steep and flat zone [15,20]. Also the data-analysis algorithms used in the commercial software are not optimized for UHMM polymers. Hence, a preliminary step of this study was the optimization of the SEC system for UHMM HA samples: on-line detectors, experimental protocol, data-analysis algorithms and column set.

Experimental protocol. With UHMM HA each detail of the SEC experimental protocol (mobile phase, flow rate, sample concentration, temperature and injection volume) have to be optimized methodically for reliable results. It is well known that the macromolecules assume more compact conformation, lower hydrodynamic radius, in θ-solvent. A possible strategy, suggested by some authors [17], could be the use of θ-solvent as mobile phase. HA in aqueous solvent is a polyelectrolyte. Theoretically a polyelectrolyte to fulfil θ-conditions needs of infinite ionic strength. Practically we have increased the ionic strength of the mobile phase from 0.15 to 0.5 M NaCl. In this way, the dimension of the HA macromolecules decreases [12] and the exclusion limit of the columns significantly increases. In general flow rate and sample concentration should be as low as possible. As flow rate we have used 0.2 ml/min. The minimal concentration depends on the sensitivity of the concentration detector. With UHMM polymer the MALS and SCV on-line detectors could use very low concentrations. Unfortunately the sample concentration reported in Table 1 were the minimal to obtain an adequate signal-to-noise ratio in the concentration detector. Finally, also the fluidic path has to be optimized. For example, we prefer to use an UV as concentration detector because its cell presents smaller flow resistance. The optimization of the SEC experimental protocol was described more in detail in a previous article [20].

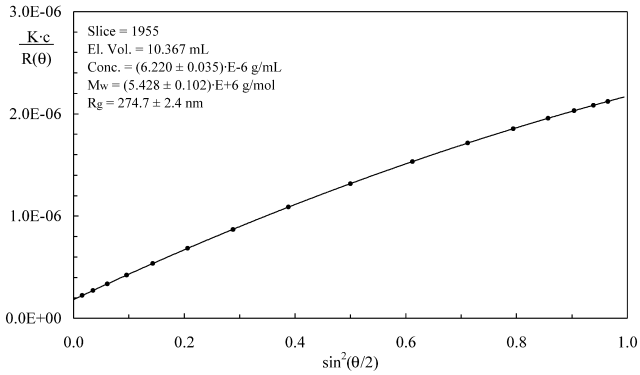

Fig. 1. Debye plot, $Kc/R(\theta)$ vs. $sin^2(\theta/2)$, for a UHMM HA fraction (slice) $M = 5.4 \times 10^6$ g/mol.

On-line detectors and data-analysis algorithms. A multi-detector SEC system do not need of calibration. Virtually, after the SEC fractionation the SEC–MALS–SCV system measures directly M_i, R_{g_i}, and $[\eta]_i$ at each elution volume. However, with UHMM HA polymers also the use of absolute on-line detectors is not deprived of problems. At each elution volume the MALS detector calculates M_i and R_{g_i}, by an extrapolation to zero angle of the reduced Rayleigh factor $R(\theta)$. Like in off-line static mode when the dimensions of the macromolecules are sufficiently large the angular variation of the scattering shows an unusual downward curvature. Fig. 1 shows the extrapolation to zero angle for an UHMM HA fraction (slice). This problem has been discussed carefully in the previous paper [12]. Both for molar mass and dimension of the macromolecules using the Zimm formalism, 2° order of polynomial fit, we have obtained the best agreement between off-line and on-line values. In the on-line MALS characterization, with regard to off-line, there is an additional problem. Usually the extrapolation to infinite dilution is neglected (the $2A_2c$ term of the MALS equation, where A_2 denotes the second virial coefficient and c the concentration). In first approximation this term is neglected because the single concentration of each slice is extremely diluted [19]. In the characterization of UHMM HA the influence of this term is not negligible. However, using the A_2 value obtained by off-line MALS it was possible to overcome this problem.

Also the on-line viscometer presents severe problems. Using the usual capillary tube (0.014 in. ID, 6 in. length) at very low flow rate, 0.2 ml/min, the apparent maximum shear-rate was approximately 750 s^{-1} and the Reynolds number was approximately 25. The shear-rate value was apparent because we do not consider the non-newtonian behaviour. In this condition we can suppose a laminar flow

also for UHMM polymers. However, the shear-rate was too high for non-newtonian solutions like UHMM HA. Using the on-line SCV detector it is relatively simple to decrease the shear-rate. We have replaced the usual capillary tube of the SCV detector with a larger internal diameter tube (0.02 in. ID, 20 in. length). In this way the average shear rate approximately decreased to 250 s^{-1}. Considering the ultra low concentration of the sample solutions after the SEC columns we can also suppose that the $[\eta]_i$ value, from SCV, was not underestimated.

Column set. The crucial point in the SEC fractionation of UHMM HA was the optimization of the column set. Obviously the performances of the SEC columns are not unlimited. Unfortunately aqueous SEC columns with large particle size (20 μm) and large pore size (higher than 4000 Å) are not commercially available. On the contrary, for organic solvents SEC columns specifically designed for UHMM polymers (20 μm particle size, 10^7 pore size) are available. We have tested four commercially available aqueous SEC columns specifically suitable for HMM polymers. The specifications of the columns (type, particle size and pore size) as described by the manufacturer was the following:

(1) 2 TSKgel from TosoHaas: G6000PW (17 μm, >1000 Å) and G5000PW (17 μm, 1000 Å);
(2) 2 PL aquagel-OH 60 from Polymer Laboratories: 15 μm, macroporous;
(3) 2 OHpak from Shodex: KB-806 (13 μm, 1000 Å) and KB-805 (13 μm, 500 Å);
(4) 2 Ultrahydrogel from Waters: 2000 (10 μm, 2000 Å) and 1000 (10 μm, 1000 Å).

At least from a qualitative point of view the $M = f(V)$ plot, from MALS, is a powerful tool for the analysis of the column set performances. Fig. 2 show the $M = f(V)$ plots obtained with the four column sets and the UHMM HA samples described previously. From up to down, from panel A to D, the column sets was, respectively, TSKgel, PL aquagel-OH, OHpak KB and Ultrahydrogel. It is well known that under equilibrium conditions, ideal SEC, the elution volume should be independent from the average molar mass of the unfractionated starting broad sample. In other words, at any elution volume should elute macromolecules with the same size (molar mass). As a result, for linear homopolymer all the $M = f(V)$ experimental plots of the different HA samples should be superimposed. Fig. 2 shows a completely different behaviour. When the average molar mass of the broad sample increases the elution of the macromolecules was delayed. Fig. 2 clearly shows the retardation, entrapment, of the macromolecules in the columns.

The retardation of the macromolecules obviously depends on the particle size and the pore size of the columns. Particle size and pore size of the four column sets used in the SEC fractionation of the UHMM HA samples

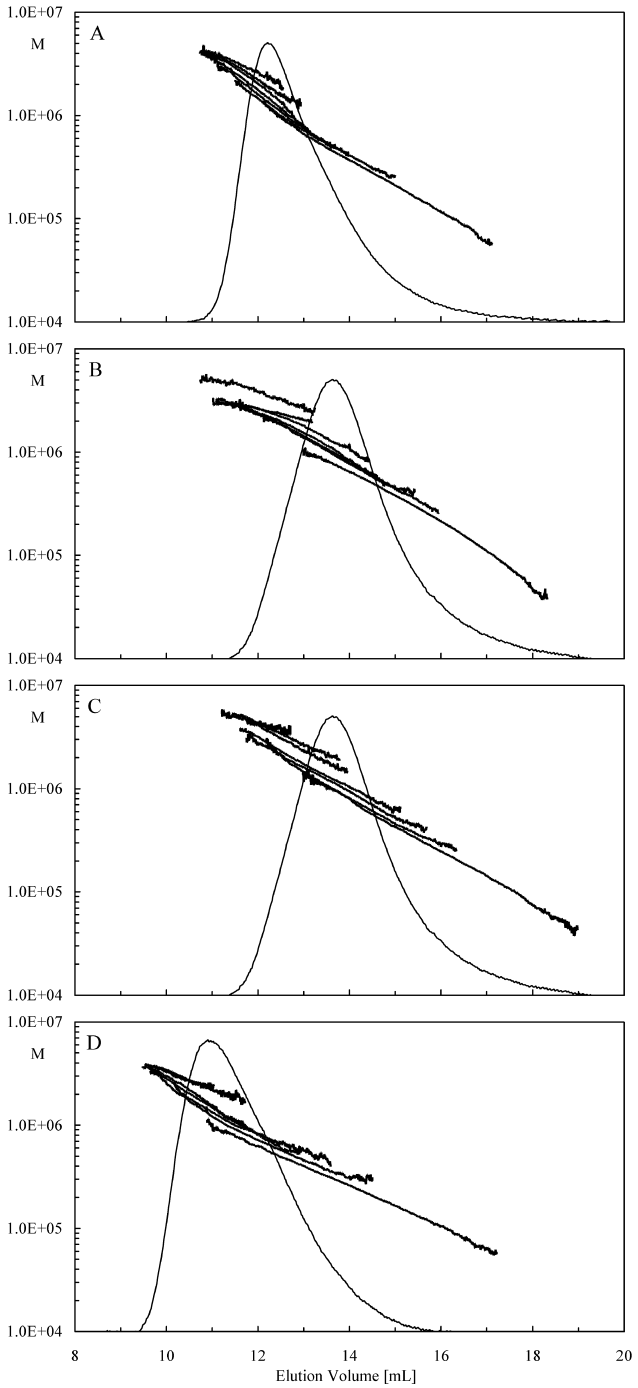

Fig. 2. $M = f(V)$ plot from on-line MALS. From up to down the column sets were, respectively, (A) TSK-Gel, (B) PL aquagel-OH, (C) OHpak KB, (D) Ultrahydrogel. M_w of the HA samples ranging from 7.4M to 0.23M g/mol.

were different. The particle size ranged from 10 μm (Ultrahydrogel columns) to 17 μm (TSKgel columns). The influence of the particle size is evident, the column set with higher particle sizes, 17 μm, shows a relatively good superimposition of the $M = f(V)$ plots up to 1.65M g/mol HA sample. On the contrary, the retardation of the higher molar mass HA samples (M_w from 3.5M to 7.4M g/mol)

were meaningful. The retardation of the macromolecules was strongly correlated with the size of the macromolecules.

The influence of the pore size on the degradation and retardation of the UHMM sample is not evident and unfortunately not generally accepted. In addition, we have to consider that the pore size data are rarely provided from the manufacturer and mainly a little reliable considering the notable difficulty of measurement. Of course, for a real fractionation the pore size should be higher than the size of the macromolecules. Unfortunately do not exist aqueous SEC columns with pore size able to fractionate higher molar mass HA samples. In a previous study, we have presented the influence of the pore size on the fractionation of UHMM HA samples [20]. In that study we have compared the performances of four Ultrahydrogel columns (10 μm of particle size) in which the average pore size were, respectively, 100, 500, 1000 and 2000 Å. When the size of the pores is small, compared to the size of the solvated macromolecules, the fractionation is a mixture of hydrodynamic and SEC. Using columns with higher pore size the fraction of macromolecules excluded from the pores apparently disappears. Using these large pore size columns only the SEC fractionation mechanism was operative. However, also for these SEC columns the retardation of the macromolecules was present. Fortunately, using MALS and SCV on-line detectors the retardation of the macromolecules does not influence the final results.

3.2. Results

When on-line detectors, data analysis, column set and experimental conditions are optimized the HA chromatograms become monomodal and substantially symmetrical. Furthermore, the experimental calibration curves ($M = f(V)$, $R_g = f(V)$ and $[\eta] = f(V)$) are deprived of waves and anomalous changes of the slope that are typical of shear degradation, viscous fingering, and in general, poor fractionation. Fig. 3 shows a three-dimensional plot of the raw signals of the MALS and concentration detectors for a HA sample with $M_w = 1.65M$ g/mol. The plot has obtained using 2 TSK-Gel PW columns and 0.5 M NaCl as mobile phase. The plot clearly shows the unusual angular variation of the scattering. Fig. 4 shows the relative experimental functions $M = f(V)$, $R_g = f(V)$, from MALS, and $[\eta] = f(V)$, from SCV. From a qualitative point of view these plots proves that using optimized experimental conditions the resolution is relatively good and the problems minimized.

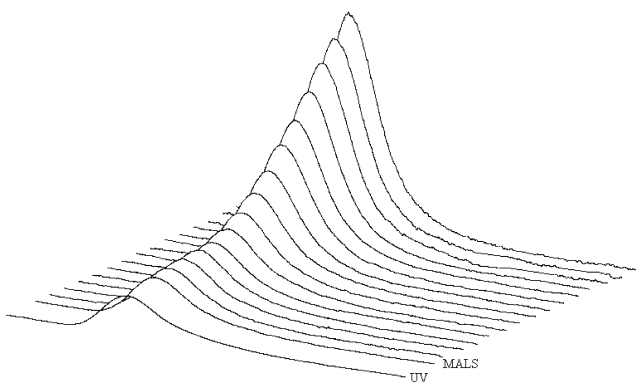

Fig. 3. Raw signals of the MALS and concentration on-line detectors for a HA sample: $M_w = 1.65M$ g/mol.

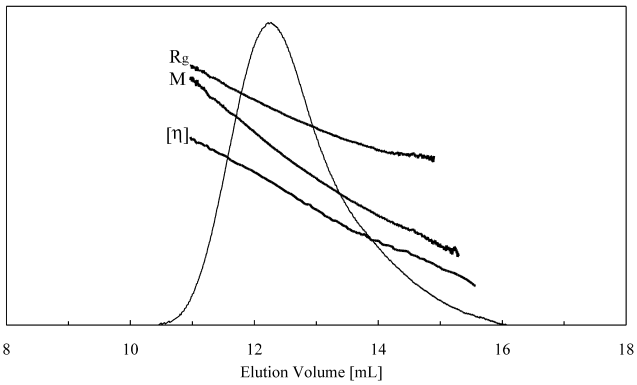

Fig. 4. $M = f(V)$, $R_g = f(V)$ (from MALS) and $[\eta] = f(V)$ (from SCV) experimental functions for a HA sample: $M_w = 1.65M$ g/mol, 2 TSK-Gel columns in 0.5 M NaCl.

Fig. 5 shows the MMD of seven HA samples, from MALS, using 2 TSKGel PW columns. The nominal M_w of the HA samples, from off-line MALS, was, respectively, $0.23M$, $0.66M$, $1.06M$, $1.65M$, $3.5M$, $5.0M$ and $7.4M$ g/mol. The relative dimension of the macromolecules R_g (z-average), in 0.5 M NaCl, approximately ranged from 50 to 330 nm. There was a very good agreement between nominal off-line M_w values, reported in Table 1, and recovered on-line M_w values up to $1.65M$ g/mol sample. The recovered on-line M_w value for the $3.5M$ sample was a little lower $3.26M$ g/mol (-6.9%). This difference gradually increases when the molar mass of the HA sample increases. The recovered on-line M_w value for the $5.0M$ and $7.4M$ samples was, respectively, $4.2M$ (-15.8%) and $5.1M$ (-32.0%). In other words the on-line M_w value agrees with the off-line value up to approximately three million of molar mass. Despite the optimization of the SEC system when the molar mass of the HA sample was approximately higher than 3×10^6 g/mol the recovered on-line M_w value was

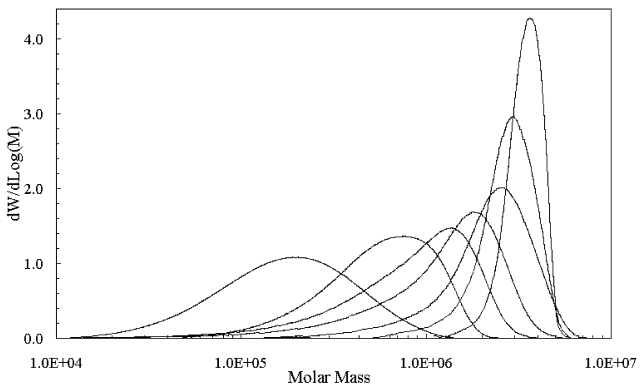

Fig. 5. MMD from MALS of seven HA samples. From left to right M_w was, respectively, $0.23M$, $0.66M$, $1.06M$, $1.65M$, $3.5M$, $5.0M$, $7.4M$ g/mol.

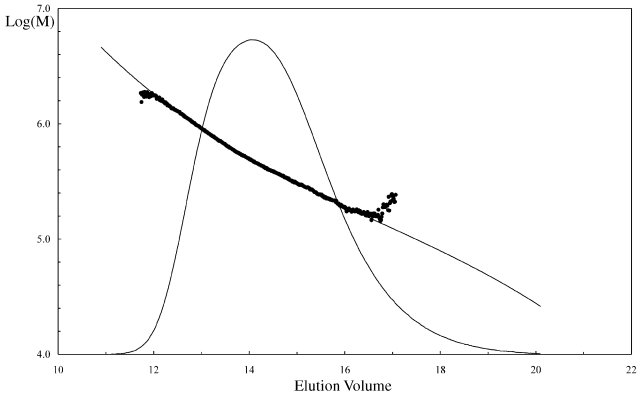

Fig. 6. $M = f(V)$ plot from on-line MALS ($M_w = 0.66M$); (●) experimental data, (—) extrapolated data.

systematically underestimated. Consequently, when the HA molar mass was ultra-high the degradation of the macromolecules in the SEC columns occurs. An estimation of the degradation of the UHMM HA samples in the columns based on the comparison of R_g or $[\eta]$ averages induces identical conclusions.

We were interested to estimate the whole MMD of the UHMM HA samples and in particular to the M_w average and the dispersity index $D = M_w/M_n$. From an accurate analysis of the MMD reported in Fig. 5 another important problem emerges. From high to UHMM HA samples the dispersity index D progressively decreases: from 1.7 (0.23M sample) to an apparent value of 1.1 (7.4M sample). More in detail D for the seven HA samples listed in Table 1 was, respectively, 1.72, 1.53, 1.44, 1.36, 1.35, 1.23, 1.12. The tendency toward a narrower MMD, when the molar mass increases is extremely evident in Fig. 5. The apparent very narrow D value is not the true value of the MMD but is an artefact. Often the D value recovered from on-line MALS detector is underestimated. Several authors impute this fact to the low sensitivity of the MALS on-line detector to the low molar mass fractions of the sample. Practically, this problem could be resolved by an extrapolation of the experimental calibration $M = f(V)$ in the tail of the chromatogram. Fig. 6 shows the $M = f(V)$ plot in which we can see both the experimental data and the relative extrapolated function. Using this extrapolated function, in the calculation of the global M_w and M_n averages, the D value effectively increases. In detail the new D values become, respectively, 2.21, 2.13, 1.57, 1.54, 1.42, 1.33, 1.25. The D values for the first two samples, 0.23M (2.2) and 0.66M (2.1), seem reasonable but the other D values, particularly the last two values, are surely underestimated. Really, this is a general problem in the use of the on-line MALS detector in the estimation of the M_n average and consequently of D. The light scattering software calculates the global M_w and M_n averages from the local values M_i and c_i (concentration) with the usual formulas assuming homogeneous fractions. It is well known that when the molar mass of the sample become ultra-high the resolution of the SEC columns dramatically decreases and the band broadening increases [14]. As a consequence at each elution volume the macromolecules are non-homogeneous in molar mass. In the presence of polydisperse fractions the MALS detector furnishes for each slice the M_w average. It is not difficult to demonstrate that if the fractions are non-homogeneous, M_{w_i} instead of M_i, the calculated global M_w average is correct but the M_n average is overestimated and consequently D is underestimated. In conclusion with UHMM samples the recovered D value from MALS requires always a critical evaluation. In base of our experience we think that the very narrow D values for HA samples reported in Ref. [21] probably are underestimated.

4. Conclusions

Static off-line methods as light scattering and viscometry could be used to obtain reliable M_w, R_g, A_2 and $[\eta]$ averages. However, these off-line methods are time consuming and not suitable for the quality control of HA. More important these off-line methods provide only average values and not the whole MMD. Therefore, we have explored the extreme potentialities of the SEC technique in the fractionation of UHMM HA. A successful SEC fractionation requires an accurate optimization of chromatographic system. Commercially available SEC aqueous columns are not optimized for UHMM HA. We have obtained our best fractionation performances using SEC aqueous columns with higher particle size and pore size and an accurate optimization of the experimental protocol. Furthermore, only MALS and viscometer on-line detectors were able to obtain reliable results though an optimization of the data-analysis algorithms needs. SEC fractionation of UHMM HA polymers was not the results of the simple equilibrium between exclusion and permeation of the macromolecules in the pores of the columns but also depends from the 'retardation'. The retardation or entrapment of the macromolecules is a very complex fractionation mechanism, it become meaningful when the size of the macromolecules is comparable with the size of the pores of SEC columns with relatively small particle size. The retardation clearly depends on the particle size of the packing and of some extent also from the pore size. Retardation is directly correlated with the sizes of the macromolecules.

Despite the difficulties a successful fractionation and characterization of UHMM HA with M_w approximately up to 3×10^6 g/mol was possible. On the contrary when the molar mass is approximately higher than 3×10^6 g/mol both the recovered M_w and D values are systematically underestimated. The extent of M_w and D underestimation increases when the molar mass of the HA sample increases.

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References

[1] Laurent TC, Gergely J. J Biol Chem 1955;212:325–33.
[2] Cleland RL, Wang JL. Biopolymers 1970;9:799–810.
[3] Cleland RL. Biopolymers 1970;9:811–24.
[4] Cleland RL. Arch Biochem Biophys 1977;180:57–68.
[5] Ribitsch G, Schurz J, Ribitsch V. Colloid Polym Sci 1980;258: 1322–34.
[6] Cleland RL. Biopolymers 1984;23:647–66.
[7] Ueno Y, Tanaka Y, Horie K, Tokuyasu K. Chem Pharm Bull 1988;36: 4971–4.
[8] Ghosh S, Reed CE, Reed WF. Biopolymers 1990;30:1101–12.
[9] Fouissac E, Milas M, Rinaudo M, Borsali R. Macromolecules 1992; 25:5613–7.
[10] Fouissac E, Milas M, Rinaudo M. Macromolecules 1993;26:6945–51.
[11] Lapcik Jr. L, Lapcik L, De Smedt S, Demeester J, Chabrecek P. Chem Rev 1998;98(8):2663–84.
[12] Mendichi R, Giacometti Schieroni A. Polymer 1998;39:6611–20.
[13] Al-Assaf S, Williams PA, Phillips GO. Proceedings of the Hyaluronan 2000 International Meeting, Wrexham, Wales, UK; 2000. p. 8.
[14] Nguyen TQ, Kausch HH. Proceedings of the International GPC Symposium'91, San Francisco, CA; 1991. p. 373–98.
[15] Mendichi R, Giacometti Schieroni A. Proceedings of the International GPC Symposium'96, St Diego, CA; 1996. p. 183–200.
[16] Chubarova EV, Nesterov VV. In: Potschka M, Dubin PL, editors. Strategies in size exclusion chromatography. ACS Symp Series, vol. 635. Washington, DC: ACS; 1996. p. 127–55.
[17] Soltes L, Berek D, Mikulasova D. Colloid Polym Sci 1980;258: 702–10.
[18] Mendichi R, Giacometti Schieroni A. In: Provder T, editor. Hyphenated and multidimensional techniques. ACS Symposium Series, vol. 731. Washington, DC: ACS; 1999. p. 66–83.
[19] Wyatt PJ. Anal Chim Acta 1993;272:1–42.
[20] Mendichi R, Giacometti Schieroni A. Proceedings of the Hyaluronan 2000 International Meeting, Wrexham, Wales, UK; 2002. p. 3.
[21] Adam N, Ghosh P. Inflam Res 2001;50:294–9.