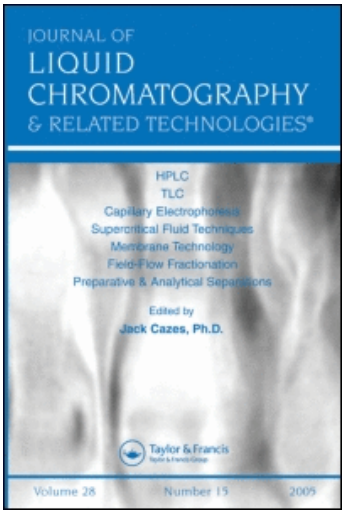

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Batch and Size-Exclusion Chromatographic Characterization of Ultra-high Molar Mass Sodium Hyaluronate Containing Low Amounts of Strongly Scattering Impurities by Dual Low Angle Light Scattering/Refractometric Detection

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Abstract: It is shown that very pure sodium hyaluronate (HA) contains small amounts of strongly scattering impurities not detectable on mass scale by refractometric detection, but clearly detectable using low angle light scattering detection during a static bulk light scattering experiment. Size filtration of its solutions does not remove these impurities, only reduces their amount depending on filter porosity. Complete removal of these particle impurities, independent of filter porosity, is achieved by hydrophobic adsorption on hydrophobic filter membranes. Using 0.1 M NaCl as a mobile phase, size exclusion chromatography (SEC) column removes the impurities by hydrophobic adsorption as well; molar masses obtained from both techniques thus agree when hydrophobic filters are used in bulk light scattering experiments. Diverse hydrodynamic flow retardation effects including slalom chromatography behavior are shown to substantially bias molar mass distributions obtained for ultra-high molar mass (UHM) HA, unless the flow rate in SEC analysis is reduced below 0.1 mL/min. Too high injected concentration (c_{inj}) is shown to introduce the onset of HA on column degradation. Correct polydispersity indices and molar mass distributions of UHM HA are obtained from SEC at a flow rate of 0.09 mL/min and optimized c_{inj}.

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Keywords: Dual low-angle light scattering/refractometric detection, Size-exclusion chromatography, Sodium hyaluronate, Static light scattering, Strongly scattering impurities

INTRODUCTION

Sodium hyaluronate (HA) is a naturally occurring, highly polydisperse negatively charged linear chain polysaccharide composed of repeating disaccharide units linked by (1-3)-β glycosidic bonds. The number of disaccharide units in a chain can reach 10^4 or more, thus yielding an ultra-high molar mass of about $4{\cdot}10^6$. The disaccharides consist of D-glucuronic acid and *N*-acetyl-D-glucosamine linked by the (1-4)-β bond. HA is present in all soft tissues of higher organisms and, in particularly high concentrations, in synovial fluid and in the vitreous body of the eye. Its excellent lubricating and water retaining properties, as well as its important role in a number of biological processes, predetermine a widespread use of HA in various medical and pharmaceutical applications.[1,2] There is a close relation between the average molar mass and molar mass distribution of HA and its application properties. Thus, in the well known application of HA in cataract surgery, an increase in average molar mass of HA was found to be beneficial.[3,4] Hence, the knowledge of both average molar mass and molar mass distribution of HA is desirable as an important parameter affecting its application performance.

Size exclusion chromatography of ultra-high molar mass (UHM) water-soluble polymers having broad molar mass distributions (MMD) is still a challenge. General obstacles to be expected here, leading among others to the loss of separation efficiency, were summarized by Giddings.[5] To find conditions for a correct SEC analysis seems impossible if the system used is equipped with a refractometric unit (DRI) only, because no narrow standards exist in this UHM range. The addition of a molar mass sensitive detector would allow differentiating between possible shear degradation and other flow rates and molar mass dependent detrimental effects. These effects should lead to distortions of the log M vs. elution volume calibration, accessible when a combination of a light scattering (LS) and DRI detection is used. The use of a SEC column set or a mixed bed column optimized to provide a linear log M vs. elution volume calibration in a sufficiently broad range of M should then facilitate the optimization of SEC conditions. The same holds for a situation where the size of a polymer coil of an UHM polymer approaches or exceeds physical maximum pore size limit of the SEC packing used. An advantage of flow field-flow fractionation (FlFFF) as a promising alternative technique to separate UHM polymers like

sodium hyaluronate[6–8] consists in the absence of this pore size limit. Nevertheless, difficulties (absent in SEC) in analyzing UHM polymers having molar mass distributions extended in M down to 10^4 were noticed,[9] and SEC still remains more rugged and seems easier to handle in developing the optimum separation conditions.[6]

Numerous recent SEC determinations (mostly using dual multi-angle light-scattering and DRI detection) of molar mass distributions of UHM HA still seem to be more or less biased by some non-size separation effects.[10–14] Typically, too low polydispersity indices M_w/M_n (sometimes as low as 1.1) are obtained. Log M vs. elution volume calibrations obtained for samples having different weight average molar mass M_w do not coincide and some on column degradation is also indicated. Some authors[12,13] of these studies are aware of a possible bias, especially in the case of low polydispersity indices because the values around 1.1, typical of specially prepared narrow standards, are non-realistic in the case of HA preparations.

A recent work dealing with distribution analysis of broad UHM poly(ethylene oxide) using SEC with dual LS and DRI detection has shown that hydrodynamic retardation phenomena and non-linearity effects provide underestimated values of M_w/M_n and introduce severe errors in the MMD unless flow rate and sample concentrations are kept at sufficiently low levels.[15] The general requirements for reliable SEC analysis of these samples resulted as follows. Injected concentration c_{inj} should be reduced to 0.1 of coil overlap concentration c^* or less and a maximum flow rate should be 6 mL/h (or less if possible). Similar or even stricter requirements should be expected to apply also in the HA case due to its semi rigid character (wormlike chain), even when high salt conditions are used to suppress its polyelectrolyte expansion in solution.[10,11] It was already shown[12] that the requirement $c_{inj} \sim 0.1\ c^*$ or less applies also to HA SEC analysis in mobile phase containing 0.15 M NaCl.

Light-scattering techniques are considered as extremely useful to detect a minor population of aggregates in polymer samples. This is certainly advantageous for aggregating systems. However, already low amounts of impurities (especially dense, i.e., strongly scattering) of comparable and larger particle size than the dissolved polymer coil can be misinterpreted as aggregates. This follows from a simple comparison of the coil segment density (macromolecule mass/coil volume) with a particle of unit density having the same hydrodynamic volume. It is well known that coil densities of linear water soluble polysaccharides are around 10^{-3} or less; using the data from ref. 16, the segment density ~ 0.0002 is obtained for HA having $M_w \sim 10^6$. The particle mass of a coil and of a dense sphere of the same diameter may thus differ by a factor of $10^3 - 10^4$. As scattering intensity is proportional to $c_i M_i$, the same difference in the scattering intensity should be expected when the polymer coil

and solid particle of the same diameter are compared. This means that already 0.1% of such dense foreign particles would increase the intensity of the scattered light by a factor of two. In fact, purity of 99.9% is a very high value for many water soluble (in particular industrial) polysaccharides. The decision whether a polymer sample contains aggregates and/or impurities on the basis of a bulk LS experiment is, hence, questionable unless some additional data supporting aggregation (or the opposite) are available. Average molar masses of water soluble cellulose derivatives obtained from static light-scattering experiments were found to be a few tens of percent higher than those obtained from SEC experiments.[17] Assuming that bulk LS experiment reflects total sample content (polymer + impurities/aggregates), a reasonable conclusion is that the SEC columns removes impurities/aggregates by adsorption. Because different adsorption behavior implies different chemical composition, it can be concluded that higher M_w values found in bulk LS experiments reflect some impurities of different composition rather than the aggregates formed from primary coils.[18] Thus, a combination of a static light-scattering experiment in bulk and SEC with LS/RI detection should be able to resolve HA aggregates and sample impurities.

The aim of this work is twofold. First, it will be shown that HA, which is probably the purest water soluble polysaccharide available, contains very low but detectable amounts of impurities visible in a bulk LS experiment. The impurities will be shown to be removable by sample filtration. Contrary to the general belief, adsorption properties of the filter material will be shown to be a decisive parameter here instead of the expected size filtration mechanism. Second, it will be shown that the SEC column also removes the impurities by adsorption and the use of very low flow rates in SEC experiments, together with low-angle light-scattering (LALS)/RI detection is necessary to obtain non-biased MMD data.

EXPERIMENTAL

Materials

Two used commercial HA samples (Sigma Chemical Company) from *Streptococcus equi* (HA1) and *rooster comb* (HA2) were recently shown[13] to be fairly pure; the amount of diverse impurities (proteins, endotoxins, RNA, and DNA) did not exceed 0.1%. Ingela Hillang from Pharmacia Upjohn, Uppsala, kindly provided three low (HA3), medium (HA4), and high molar mass (HA5) samples. Their purity should be at least comparable to previous ones because of their potential ophthalmologic application.

Analytical reagent grade NaCl was obtained from Merck (Darmstadt, Germany) and used without further purification. Water was from a Millipore Milli-Q_{PLUS}^{UF} ultrapure water purification unit (Millipore Corp., Bedford, MA, USA).

SEC-LALS-RI

The modular chromatograph consisted of a Shimadzu LC-10AD*vp* pump (Shimadzu Corp., Kyoto, Japan), a vacuum degassing unit DEGA-SYS™ (Sanwa Tsusho, Ltd., Tokyo, Japan), a Pharmacia injection valve V-7 with 500 μL loop (Pharmacia & Upjohn, Uppsala, Sweden), a Chromatix KMX-6 LALS detector (LDC/Milton Roy, Sunnyvale, CA), and a Waters 2410 differential refractometer (Waters Assoc., Milford, MA) connected through a Black Star (Huntingdon, UK) 2308 A/D converter to an IBM compatible computer. Online RI-LALS arrangement allows the simultaneous determination of M and c at any elution volume ("slice"). The following relationship is valid for Rayleigh scattering from a polydisperse polymer/solvent system at low angle (6-7°):

$$\frac{(K^* c)}{R_\Theta} = \frac{1}{M_w} + 2A_2 c \tag{1}$$

where c is the concentration of scattering species, R_Θ is the excess Rayleigh scattering factor, M_w is the weight average molar mass of scattering species and A_2 is the second virial coefficient. $K^* = 4\pi^2\nu^2 n^2/(N_A\lambda^4)$ where n is the refractive index of the solvent, λ is the wavelength *in vacuo* (633 nm), N_A is the Avogadro constant and ν is the refractive index increment of the scattering species in the mobile phase. The angular dependence of the scattered light is omitted at the low angle used. The value[16] $\nu = 0.150$ was used here. If correct separation takes place, the polymer observed in a slice is assumed to be uniform. Polydispersity and column band broadening dilutes the sample considerably; hence, the term A_2c can be mostly neglected if the concentration of the injected solution is sufficiently low. Conventional calibration log$M$ vs. elution volume (V_e) is thus directly obtained. Homemade software (M. Netopilík, Institute of Macromolecular Chemistry) allows online data accumulation and all calculations of molar mass distributions and their averages. A TSKgel GMPW linear (7.5 × 600 mm) column, particle size 17 μm, (Watrex, Prague, CR) was used. An in-line mobile phase filter holder with 0.025 μm membrane (VSWP 25 mm, mixed cellulose esters, Millipore) was positioned between the pump and injection valve. Aqueous sodium chloride (0.1 M) was used as a mobile phase in all experiments. No post-column filter was between the column and LALS detector.

Flow Injection System

A simple transformation of the above SEC setup was accomplished using a Teflon capillary (length 60 cm, inner diameter 0.5 mm) instead of the SEC column. Because the loop injection in the absence of a SEC column gives bad double peak shapes with long tailing for UHM polymers,[19] a 10 mL Superloop (Pharmacia & Upjohn, Uppsala, Sweden), which acts as a mobile phase driven syringe, was mounted instead of capillary loop. The injected volume was usually 4 mL; hence, this modification allowed producing almost rectangular concentration pulses visible by both detectors. The frequently used[20] short low porosity column was not used here because of its possible adsorption interactions with unknown potential impurities.

Preparation and Filtration of HA Solutions

Stock HA solutions, 0.1 wt. %, in mobile phase were prepared (48 h dissolution, gentle mixing) and diluted (gentle mixing, 3 h) to the injection concentration. Sample filtration prior to injections was performed using a "Genie" programmable syringe pump (Kent Scientific Corporation, Torrington, CT, USA) at a flow rate of 0.25 mL/min. Hydrophilic PVDF membrane filters having porosity 1 μm (Puradisc^{TM}13 mm, Whatman, Maidstone, UK), 0.45 μm (Millex HV_{13}, Millipore), and 0.22 μm (Millex GV_{13}, Millipore), mixed cellulose esters (MCE) membrane filter 0.8 μm (Millex AA Millipore), and hydrophobic Teflon membrane filters with porosity 1 μm (Puradisc^{TM} 13 mm, Whatman, Maidstone, UK) and 0.5 μm (Millex FH_{13}, Millipore), served for sample filtration. Teflon filters were always wetted with methanol and rinsed with mobile phase before use. Because HA always contains a significant amount of water, the water content in the samples was checked by the standard Karl-Fischer titration, modified carbazole reaction,[21] and calculated using sample recovery values from chromatography experiments. All three techniques generally agreed within 3%, confirming the reliability of $\nu = 0.150$ used in calculations of true HA concentrations from RI data.

RESULTS AND DISCUSSION

Flow Injection Experiments

The advantages of the use of flowing arrangements for measurement of light-scattering in systems containing polymer coils and coexisting particles was well documented.[22,23] In particular, signal spikes resulting from

the flowing particles are superimposed here over a constant background LS signal resulting from polymer coils. Simultaneous particle counting and polymer molar mass determination are thus feasible.

A small amount of strongly scattering particles in HA1 sample as observed by the LALS detector is displayed in Figure 1a. A dramatic difference between filtration through a hydrophilic PVDF and hydrophobic PTFE filter of the same pore size is a clear evidence of another mechanism than size filtration. A natural conclusion is that impurities being hydrophobic are adsorbed only on PTFE filter membrane and their adsorption is independent of the pore size of the PTFE filter used. Accordingly, using a PTFE filter having 0.5 μm pore size instead of 1 μm, LALS curve indistinguishable from that one displayed in Figure 1a (1 μm PTFE filter) was obtained. Also, MCE 0.8 μm membrane filter was checked expecting its intermediate hydrophobicity between PTFE and PVDF membranes. A complete removal of impurities as observed with PTFE filters was found also in this case. On the other hand, the use of a hydrophilic PVDF filter with a smaller pore size (0.45 μm) resulted in partial reduction of spiking when compared with a 1 μm PVDF filter, indicating partial size filtration in this case. A significant contribution to the LALS

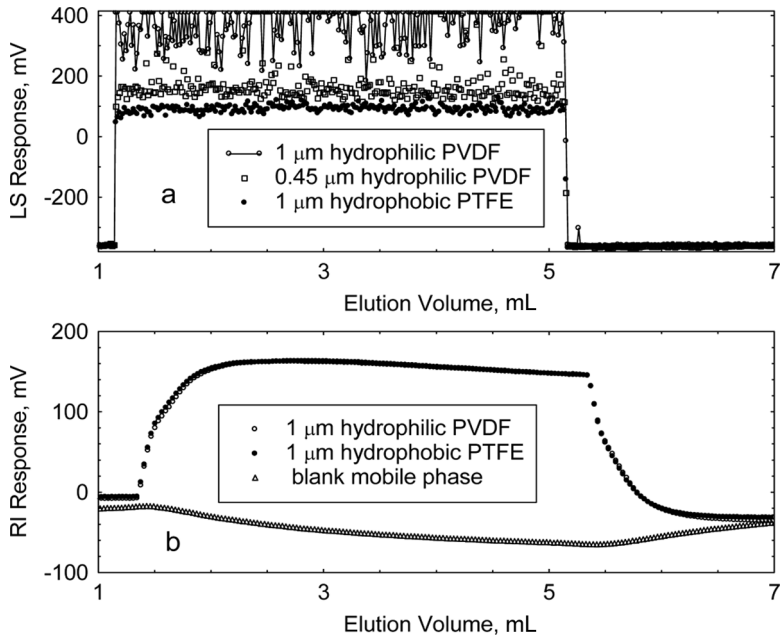

Figure 1. Flow-injection LALS (a) and RI (b) responses of HA5 sample filtered through adsorbing (PTFE) and non-adsorbing (PVDF) filters.

signal still persisted here when compared with a 1 μm PTFE filter (Figure 1a).

Contrary to the LALS signal, the corresponding RI signals (Figure 1b) reflecting the sample mass coincided for PVDF, MCE, and PTFE filters. This means that the mass of removable impurities is not detectable on the mass scale used. It should be noted that the shape difference between RI and LALS signals in Figure 1 follows from the effect of dissolved air in very dilute non-degassed sample solutions (25 μg/mL) as evidenced by the injection a of non-degassed mobile phase in Figure 1b.

All five HA samples behaved in a similar way (pronounced spiking and increase in LS signal with PVDF filters in contrast to PTFE filters and coincidence of RI signals) in these experiments, indicating very low mass contamination by strongly scattering impurities. To further support this conclusion, successive injections of 8 mL of 0.04% HA3 solution were performed using a single 1 μm PTFE sample filter. Neither spiking signs nor indications of filter blockage were observed after 8 injections (total 26 mg HA3). Taking into account the small size of filter membrane, which defines its active surface, the mass of removed material must be very small. It was of interest to see changes of the LALS signal using a smaller PVDF filter size because the resulting loss of mass of an UHM HA sample was already observed.[7] When a PVDF filter with pore size 0.22 μm was used for sample HA1, some sample loss was found indicating the onset of size retention of HA. Sample HA3 was found to be filterable through the pore size 0.22 μm without any sample loss. Figure 2 clearly shows that even this filter does not remove all impurities. This implies that a small bias of M would persist even in this case, due to the presence of strongly scattering particles smaller than 0.22 μm. Molar masses of HA 1–5 were calculated from experiments using both 1 μm PVDF and PTFE filters. According to common practice, the minimum LS points were taken for these calculations to reduce the effect of spikes in the case of PVDF filters. Although second virial coefficient is usually taken as a second order term (Equation 1) in dilute solution scattering, the value $A_2 = 0.002$ (determined for sample HA 1 in 0.1 M NaCl) was used as an average value for all broad HA samples investigated and used in all calculations. Compared with other water soluble polymers, this value is exceptionally high and cannot be neglected against experimental error in bulk experiments in Table 1. Only a small dependence[16] of A_2 on M was neglected in the evaluations of both bulk and SEC data discussed below. The calculated M_w values are presented in Table 1 and compared with the results obtained from SEC measurements. The content of impurities as observed by LALS detection using a PVDF filter is rather similar among the samples, with the exception of HA5, which appears surprisingly clean. Contrary to flow injection experiments; SEC results did not depend on the choice of injection filter, hence, the column itself removed

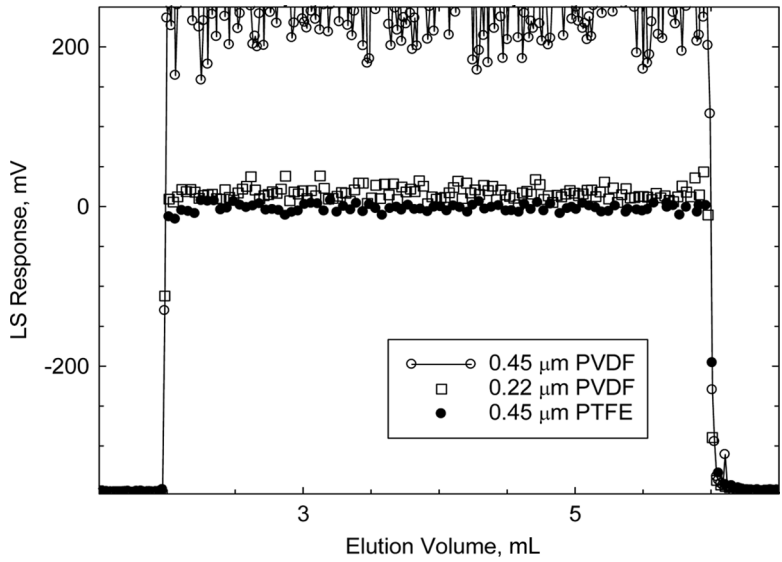

Figure 2. Flow-injection LALS responses of HA3 sample filtered through adsorbing (PTFE) and non-adsorbing (PVDF) filters.

the impurities in the same way as the PTFE filter in bulk experiment. Nevertheless, the use of injection PTFE filters is beneficial also in SEC because it prevents the column from contamination with unknown heterogeneous particles. These particles may stay or move through the column in an unpredictable way and sometimes may lead to unexpected difficulties with the LS baseline. The agreement of the M_w values from bulk and SEC experiments suggests that almost all removed impurities

Table 1. Comparison of M_w of HA samples obtained from batch experiments using 1 μm PTFE and PVDF filters with SEC results at 0.09 ml/min

	Superloop			SEC[a]		
Sample	c_{inj} μg/mL	M_W^a	M_W^b increase (%)	c_{inj} μg/mL	M_W^a	M_w/M_n
HA 3	21.2	562 000	35.3	50.5	555 200	4.7
HA 2	19.6	1 076 000	28.0	35.4	1 029 000	6.7
HA 1	21.6	1 523 000	39.5	20.1	1 518 000	7.1
HA 4	17.4	2 425 000	52.5	14.6	2 432 000	5.4
HA 5	10.0	4 291 000	1.3	17.1	4 395 000	3.2

[a]1 μm PTFE sample filter.
[b]1 μm PVDF sample filter.

are strongly hydrophobic because the column used is assumed to be hydrophilic, exhibiting only mild hydrophobic interactions. Summarizing, it can be stated that all general adsorption chromatography principles should apply also to hydrophobic filters if the amount of hydrophobic impurities is low enough.

SEC-LALS-RI

Having experience with SEC of UHM poly(ethylene oxide),[15] a pronounced effect of flow rate was anticipated also here. Figure 3 shows variations of both LS and RI signals with flow rate for HA5 having the highest M_w. A significant shift of both signals toward larger elution volumes and peak shape changes are visible between 0.09 and 0.18 mL/min. Going from 0.18 mL/min to 0.333 mL/min, only a moderate change of RI signal but a persisting change of LS signal indicates an additional shift of the largest macromolecules along the elution volume axis. The primary data from Figure 3 were recalculated to the log $M = f(V_e)$ calibrations (Figure 4a). This picture clearly shows that the

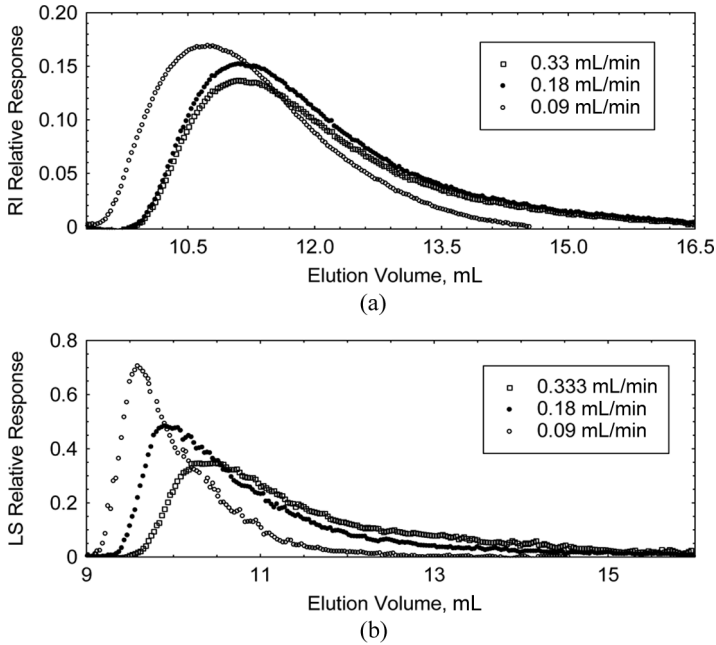

Figure 3. SEC RI (a) and LALS (b) responses of HA5 sample as a function of flow rate.

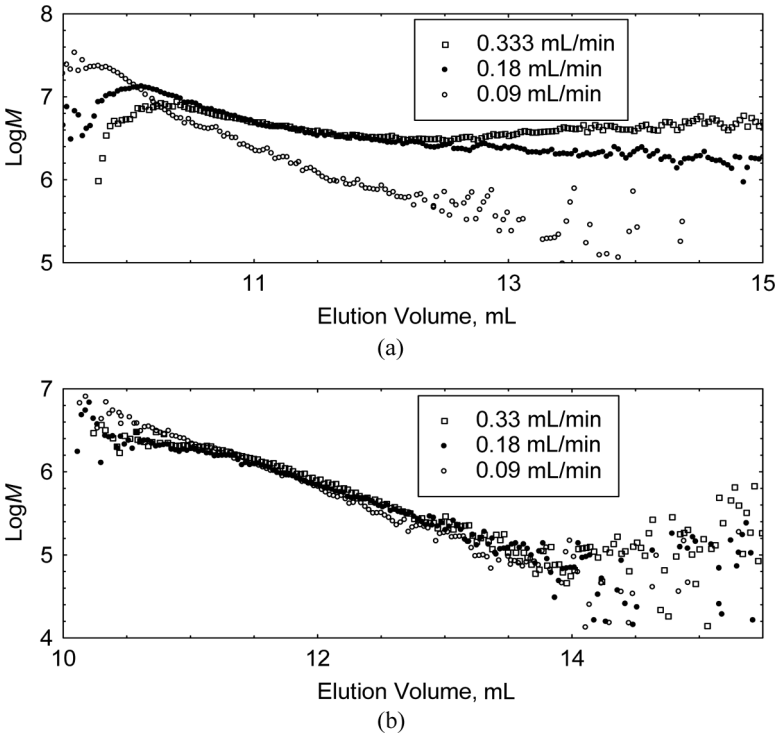

Figure 4. Log$M = f(V_e)$ calibrations obtained for samples of HA5 (a) and HA3 (b) as functions of flow rate.

flow rate 0.18 mL/min substantially reduces SEC separation, whereas 0.333 mL/min cancels it almost completely. As expected, this effect was stronger the higher was the M_w. The log $M = f(V_e)$ calibrations obtained for the HA3 sample having the lowest M_w are displayed in Figure 4b using the same resolution on Y axis for comparison with HA5. Only a small effect of flow rate is observed when sample M_w sufficiently decreases. Table 2 summarizes the effect of flow rate for both HA3 and HA5 in terms of M_w, and M_w/M_n. M_w is always correct here (being in principle the ratio of the LS and RI peak areas multiplied by instrumental constants), but M_n value is biased. These results clearly show that the frequently observed low M_w/M_n (the lower the higher are M_w) in dual LS/RI SEC of HA are artifacts related to the hydrodynamic retardation phenomena[5] at too high flow rates. An additional purely hydrodynamic retardation effect, called slalom chromatography,[24] was described in hydrodynamic chromatography (HDC) separation studies of plasmid DNA. When a certain flow rate was exceeded, large polymer coils were

Table 2. *M_w and M_w/M_n* values for HA 5 and HA 3 obtained by SEC at various flow rates

	HA 5[a]		HA 3[b]	
Flow rate mL/min	M_w	M_w/M_n	M_w	M_w/M_n
0.090	4 395 000	3.2	555 200	4.7
0.180	4 329 000	1.3	562 600	2.7
0.333	4 335 000	1.1	547 300	3.2

[a] $c_{inj} = 17.1\,\mu g/mL$.
[b] $c_{inj} = 50.5\,\mu g/mL$.

entangled, oriented parallel to flow lines, and began to move through much narrower parts of the packed bed. The longer the macromolecule chain, the more difficult it was to pass through these interparticle channels and the elution order opposite to that in SEC was observed. This effect seems to be a general one because it was recently proved[25] to operate even during HDC of UHM polystyrenes on non-porous column packing in THF. HA coils are known to be very easily entangled;[1] hence, such behavior should apply also here and should be added to the group of retardation phenomena mentioned above. Our data confirm net retardation; nevertheless, this does not mean the absence of an HDC contribution (acceleration mode) for very large coils. All these effects are flow rate dependent and increase with molar mass. Their possible concentration dependence should be mentioned as well; the on column dilution of broad samples due to separation is very large. A very complex picture thus results for broad HA samples. Moreover, mixed modes of separation (HDC/slalom, HDC/SEC, and SEC/slalom) should be expected to operate to some degree. It is quite clear that the dominant mechanism leading to the observed retardation is impossible to distinguish. Perhaps, a much more pronounced shift of both LS and RI signals in Figure 1, when compared with UHM PEO behavior in the previous work,[15] might indicate greater impact of the slalom chromatography effect here.

The log $M = f(V_e)$ calibrations obtained at 0.09 mL/min for all the HA samples investigated are presented in Figure 5. Calibrations obtained for HA 1–4 are linear and coincide within the experimental error, indicating that pure SEC is operative for these samples up to $M \sim 10^7$at flow rate 0.09 mL/min. The lower part of the calibration obtained for HA5 falls on the common straight line as well, but onset of some deviation appears above $M \sim 10^7$. A further reduction of flow rate to 0.068 mL/min in SEC experiment did not improve the shape of this calibration; only increased LS noise and more serious difficulties with RI baseline

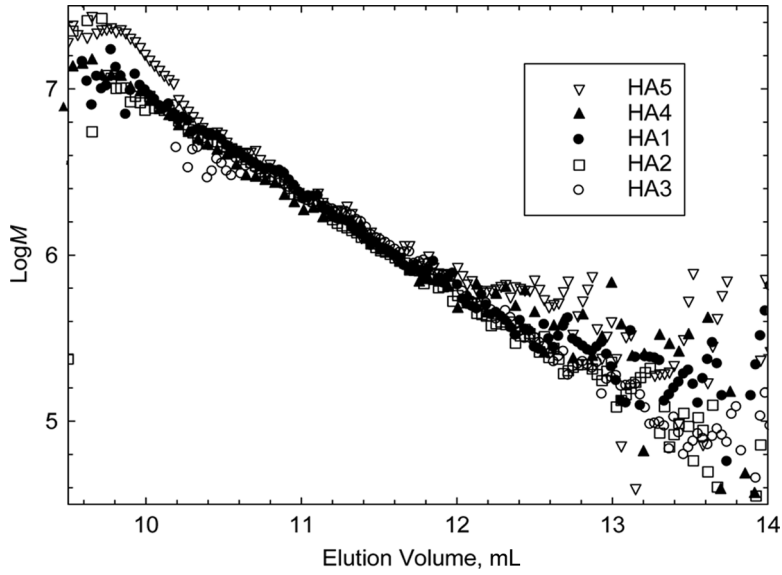

Figure 5. Log$M = f(V_e)$ calibrations obtained for samples of HA1–HA5 at flow rate 0.09 mL/min.

resulted. It seems that the deviation around $M \sim 10^7$ and above indicates that the largest components of HA5 exceed the exclusion limit of the column used. It should be noted that the shape of log $M = f(V_e)$ curve obtained for HA5 is similar to its predicted shape in the case when both SEC and HDC modes operate.[26] The HDC mode having a lower resolution than SEC mode should then introduce additional separation of coils having diameter exceeding the maximum SEC pore diameter. Such separation contribution might then appear in Figure 5 as the observed deviation around and above $M \sim 10^7$.

The agreement of M_w values obtained from flow injection and SEC measurements at c_{inj} in Table 1, indicates here the absence of frequently discussed shear degradation of UHM HA during SEC analysis.[5,12] The same conclusion follows from Table 2, where M_w values remain constant within the experimental error at c_{inj} used irrespective of changes in flow rate. Shear stress (shear rate multiplied by viscosity) as a decisive degradation parameter,[5] increases at higher flow rates and/or sample concentrations. Owing to on column dilution (here around ten) shear stress considerably decreases when the injected sample zone moves down. Thus, the highest shear stress should be expected to operate in the initial phase of separation near to the column top. Flow rate and c_{inj} were varied during SEC of HA 1 (Table 3). It follows from the Table that the increased c_{inj} has a dominant degradation effect at both flow rates. A practical

Table 3. M_w a M_w/M_n values of sample HA 1 obtained from SEC at various c_{inj} and flow rates

	Flow-rate, mL/min			
	0.09		0.18	
c_{inj} μg/mL	M_w	M_w/M_n	M_w	M_w/M_n
81.1	1 174 000	5.6	1 138 000	2.9
42.6	1 360 000	6.1	1 265 000	2.8
20.1	1 518 000	7.1	1 539 000	2.2

conclusion here is that larger injection loops allowing additional reduction of c_{inj} should be advantageous in the case of UHM polymers.

Molar mass distributions of all five HA samples investigated are presented in Figure 6. As expected, all distributions have a similar shape, are broad covering more than three decades of M, and contain detectable amount of molecules having M below 10^4. Linear and common calibrations log $M = f(V_e)$ obtained at 0.09 mL/min in Figure 5 can be taken as the evidence that distributions of HA 1–4 are not biased. A small bias of HA5 distribution has to be accepted around and above M ca 20 000 000 because the straight line fit of log $M = f(V_e)$ used to calculate distribution

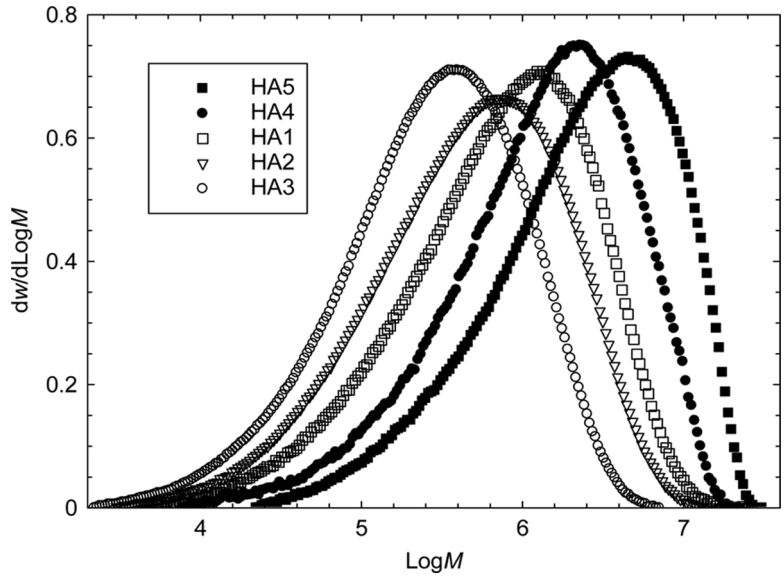

Figure 6. Molar mass distributions of HA1–HA5 obtained by SEC at flow rates 0.09 mL/min and optimized c_{inj}.

somewhat underestimates the largest macromolecules. Using the power law dependence $R_g = f(M)$ found[27] for HA, the diameter $2R_g = 1.24\,\mu m$ for $M = 20\,000\,000$ can be calculated. This value already seems to exceed a physical pore size limit of the column used. It is hard to accept that those wider pores capable of accommodation of larger coils may exist in any of the available SEC column packings. The combination of SEC (reflecting better low M tail of the distribution) and FlFFF (reflecting better UHM tail of the distribution) should be a perfect solution in this case.

CONCLUSIONS

A very small amount of strongly scattering particle impurities present in HA samples can be clearly detected in flow injection (batch) RI/LALS experiment with LALS detection, owing to its extreme sensitivity to the presence of such particles, but cannot be found by RI mass detection. Contrary to the common belief, these impurities are difficult to remove from UHM HA by size filtration prior to batch LS experiment. Alternatively, their adsorption to the hydrophobic filter surface, independent of filter pore diameter, can be used to remove them completely.

These impurities do not affect the results of SEC analysis of HA in 0.1 M NaCl because the column known to exhibit mild hydrophobic interaction traps these impurities as well. Diverse hydrodynamic flow retardation effects including the slalom chromatography behavior are shown to substantially bias MMD results obtained for UHM HA, unless the flow rate in the SEC experiment is reduced below 0.1 mL/min. A too high c_{inj} is shown to introduce the onset of HA on column degradation. The correct polydispersity indices and molar mass distributions of UHM HA are obtained from SEC at flow rate 0.09 mL/min and optimized c_{inj}.

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