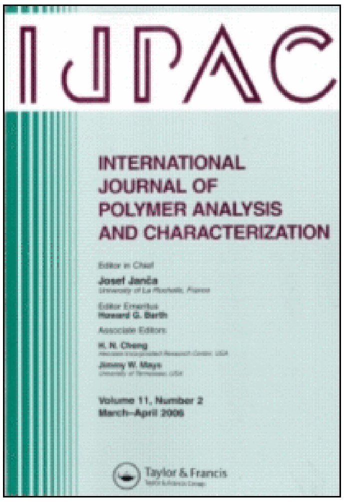

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Use of Steric Exclusion Chromatography to Characterize Hyaluronan, a Semirigid Polysaccharide*

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This paper concerns the mechanism of separation of polyelectrolytes by steric exclusion chromatography (SEC). It is demonstrated that the fractionation follows universal calibration when the electrostatic exclusion effect, that is, the long-range electrostatic repulsions, are screened; a minimum concentration of 1–1 electrolyte around 10^{-2} M is necessary. In addition, SEC with multidetection is convenient to fully characterize a polyelectrolyte and to demonstrate the role of the ionic concentration on chain dimensions and to determine the intrinsic persistence lengths, a convenient characteristic for polysaccharides, which are considered as wormlike chains. The analysis is developed on a sample of hyaluronan.

Keywords: Polyelectrolyte; Hyaluronan; SEC; Electrostatic repulsion; Persistence length; Second virial coefficient

INTRODUCTION

The behavior of charged macromolecules has been discussed for a long time. The existence of electrostatic interactions causes a large influence of the polymer concentration and/or external salt concentration on

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their solution properties, especially on viscosity. These effects are due to modification of chain expansion and are reflected by change of the radius of gyration $\langle \rho^2 \rangle^{1/2}$ of the chain or intrinsic viscosity $[\eta]$.[1–4] At the same time, the second virial coefficient A_2, which measures the excluded volume between interacting particles, varies as a function of electrostatic repulsion between polyelectrolyte chains, which is directly related to the screening effect caused by the salt. The change of $\langle \rho^2 \rangle^{1/2}$, $[\eta]$ and A_2 was recently discussed by Reed[1,2] and the experimental data were compared with theoretical prediction including electrostatic persistence length and electrostatic excluded-volume theories with no adjustable parameter. The same approach was also used to analyze the data of hyaluronic acid (sodium form) obtained by static measurements of light scattering and viscometry[3] or by steric exclusion chromatography (SEC) with multidetection. Other ionic polysaccharides, especially xanthan,[4] were examined using this treatment. The analysis proposed is based on a wormlike chain model in which the intrinsic persistence length L_p plays an essential role and characterizes the properties of the polymers; the recent values obtained for L_p of hyaluronan (HA) are given in the range 70–80 Å.[5,6]

Considering the characteristics of the polymeric chain and taking into account the interchain interactions discussed for viscosity[7,8] or combining static light scattering and SEC experiments, the mechanism of SEC separation of water-soluble ionic polymers will be discussed and demonstrated with the behavior of hyaluronan.

EXPERIMENTAL

The sample of HA was obtained from ARD Company (Pomacle, France). It was purified and isolated in its sodium form by the usual method.[9] HA was partially depolymerized by sonication to decrease its hydrodynamic volume for SEC analysis.

The static measurements of M_w and A_2 were performed with a multi-angle laser light scattering detector at the wavelength 633 nm (Wyatt DAWN DSP-F, Santa Barbara, CA, USA) at 30°C; the dn/dc was 0.144 mL/g in 0.1 M NH_4NO_3. All solutions were clarified before analysis by filtration through porous membranes (0.2-μm pore diameter) from Millipore (Bedford, MA, USA). The intrinsic viscosity was obtained using a Low Shear 30 (Contraves, Zürich, Switzerland) to obtain the Newtonian plateau viscosities and test for shear-rate effects. The characteristics of the HA sample used in this work were $M_w = 440{,}000$; $M_w/M_n = 1.61$; and $[\eta] = 876\,\mathrm{mL/g}$ in 0.1 M NaCl. Viscosities of HA solutions were obtained using a Low Shear 30 viscometer for shear rates lower than $10^2\,\mathrm{s}^{-1}$ and a Carri-med CS50 rheometer (Dorling, Surrey, England) for shear rates higher than $10^2\,\mathrm{s}^{-1}$.

The SEC experiments were performed at 30°C using a Waters AC 150 chromatograph (Milford, Mass., USA) equipped with three on-line detectors: a differential refractometer, custom-made capillary viscometer, and MALLS (Wyatt Technology). Two columns were used in series: Shodex OH-Pack 804 and 805 (Showa Denko, Tokyo, Japan); the eluent was 0.1 M NH_4NO_3 protected with 0.2 g/L NaN_3.

RESULTS AND DISCUSSION

SEC of a semirigid polymer, especially a charged polymer, allows for the determination of the values of $\langle \rho^2 \rangle^{1/2}$ and $[\eta]$ as a function of the polymer molar mass M for different salt concentrations of the eluent c_s, in addition to the molecular weight distribution.

Influence of c_s on the Radius of Gyration

An example is given in Figure 1 for a salt concentration varying from 3×10^{-1} to 10^{-2}M NH_4NO_3; the radius of gyration is given as a function of the molar mass allowing the determination of the parameters K' and ν in different salts in the following relationship:

$$\langle \rho^2 \rangle^{1/2} = K' M^{\nu}. \qquad (1)$$

In Figure 1, the experimental data (solid line) are compared with the calculated values (crosses) using the method previously described.[4] This method is based on a wormlike chain model in which the stiffness is characterized by an intrinsic persistence length L_p modified by electrostatic repulsion contribution L_e; the total persistence length being $L_T = L_p + L_e$.

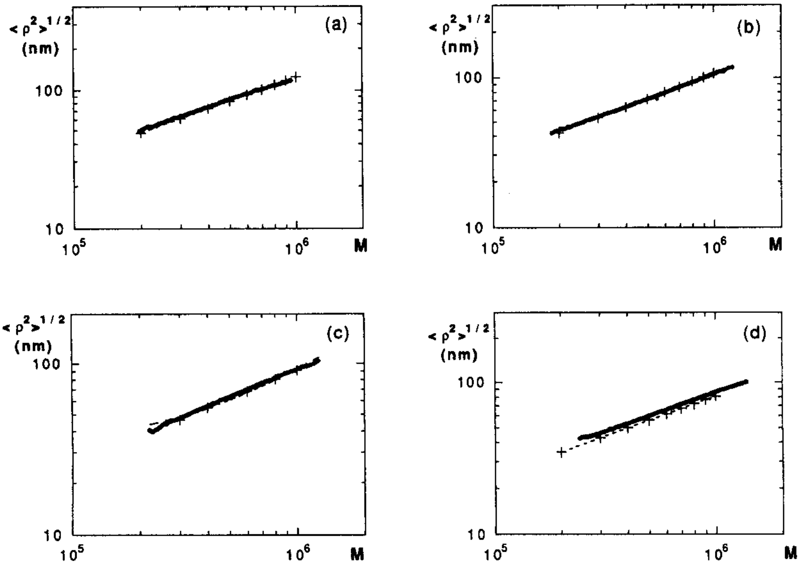

FIGURE 1 Log–log plot of the radius of gyration $\langle\rho^2\rangle^{1/2}$ (nm) versus the molecular weight for different salt concentrations, in which the experimental data (solid lines) are compared to theoretical values (crosses) obtained by setting $L_p = 70$ Å. (a) NH_4NO_3 10^{-2} M; (b) NH_4NO_3 3×10^{-2} M; (c) NH_4NO_3 10^{-1} M; (d) NH_4NO_3 3×10^{-1} M.

The second contribution coming from long-range electrostatic repulsion is the electrostatic excluded-volume characterized by an expansion parameter α_s calculated from Fixman and Skolnick.[10]

If $L_T \ll L$,

$$\langle\rho^2\rangle_{cs} = \left[\frac{LL_T}{3} - L_T^2 + 2\frac{L_T^3}{L} - 2\left(\frac{L_T^4}{L^2}\right)[1 - \exp(-L/L_T)]\right]\alpha_s^2 \qquad (2)$$

where α_s is the electrostatic excluded-volume expansion parameter at a given ionic concentration c_s, assuming that the excluded-volume expansion parameter at infinite ionic concentration tends to 1, and L is the chain contour length.

Figure 1 shows good agreement between the two series of values when the intrinsic persistence length is fixed at 70 Å. This implies *a posteriori* the validity of the Odijk treatment which assumes that L_e varies as c_s^{-1}.[11] The values of L_e and the parameter ν are given in Table I. The proposed treatment allows the prediction of the modification of $\langle\rho^2\rangle^{1/2}$ with ionic concentration and the molar mass directly related to the contour length L.

TABLE I Characteristic parameters for the relationships $\langle\rho^2\rangle^{1/2}(M)$ and $[\eta](M)$* at different salt concentrations

$[NH_4NO_3]$ mol/L	ν *Calculated*	*a Calculated*	L_e (Å)
3×10^{-1}	0.53	0.70	0.53
10^{-1}	0.56	0.78	1.6
3×10^{-2}	0.58	0.85	5.3
10^{-2}	0.59	0.90	15.9
3×10^{-3}	0.6	0.97	52.8

*Molecular weight range: 2×10^5–2×10^6.

Variation of A_2 with c_s

Following Reed,[1] one assumes that A_2 for a polyelectrolyte can be approximated as the sum of $A_{2,\mathrm{el}}$ (due to electrostatic) and $A_{2,\mathrm{HS}}$, the contribution to the excluded volume obtained by extrapolation of A_2 to infinite c_s corresponding to the screening of electrostatic interactions.

From Yamakawa,[12] the perturbation theory of excluded volume gives

$$A_{2,\mathrm{el}} = \left(N_a N_k^2 \beta / 2M^2\right) h_0(\bar{Z}) \tag{3}$$

with N_k the number of Kuhn segments, N_a Avogadro number and

$$h_0(\bar{Z}) = \left[1-(1+3.9\bar{Z})^{-0.468}\right] \Big/ 1.83\bar{Z} \tag{4}$$

in which $\bar{Z} = Z/\alpha_s^3$; Z, β, α_s are obtained from the treatment of $\langle\rho^2\rangle$ and defined elsewhere.[1]

The values of A_2 were obtained from SEC when we imposed that M_w, obtained from the integral of the light scattering signal after correction for A_2 and from the calculated injected mass determined from the integral of the refractive index signal after correction for d*n*/d*c*, must remain constant for different injections of the same amount of polymer at different salt concentrations; the reference is given by static light scattering measurements determined using the usual Zimm plot method. The data are given in Figure 2. The extrapolation to infinite c_s gives $A_{2,\mathrm{HS}} = 1.01\times10^{-3}$ $(\mathrm{mol\cdot cm^3\cdot g^{-2}})$ which means that, under these conditions, the solubility of this hydrophilic polymer remains relatively good in the absence of electrostatic repulsion. The calculated values are compared with the experimental values in

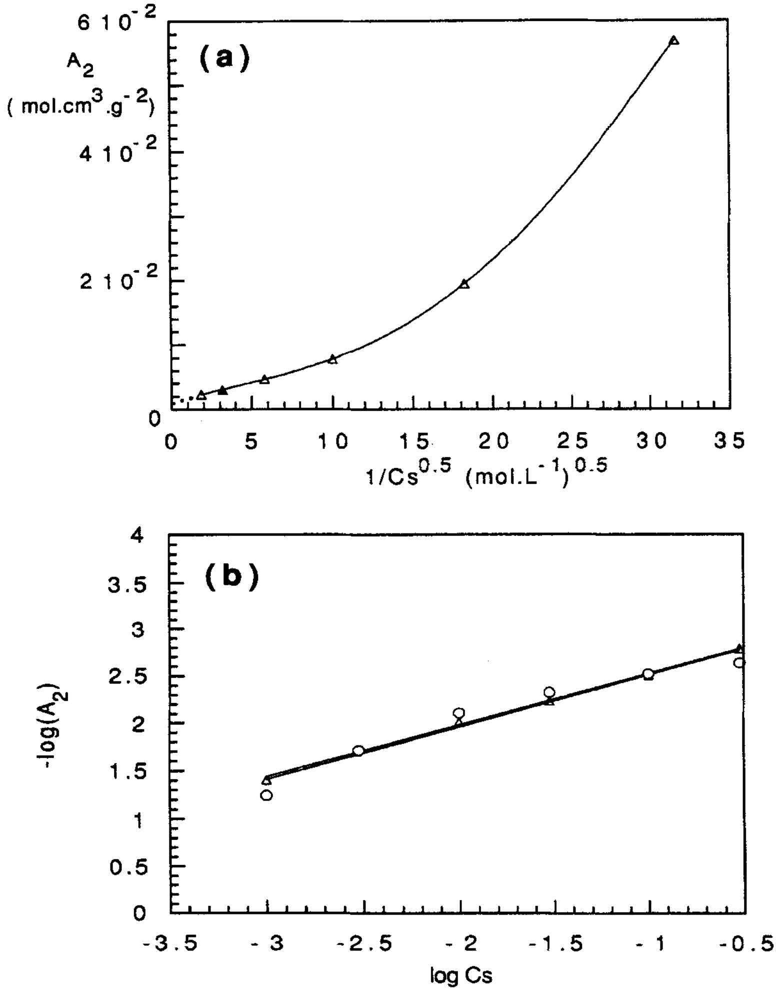

FIGURE 2 Variation of the second virial coefficient as a function of the salt concentration. (a) Variation of A_2 as a function of $c_s^{-1/2}$. (▲) Static light scattering measurements; (△) SEC experiments; (b) Comparison between experimental data (○) and calculated values (△).[1]

Figure 2(b); the agreement is very good and the results demonstrate clearly the large role of electrostatic interactions (the value of $A_{2,\mathrm{HS}}$ is low compared to the electrostatic contribution). Our conclusion confirms that of Reed[1] and seems to validate this approach.

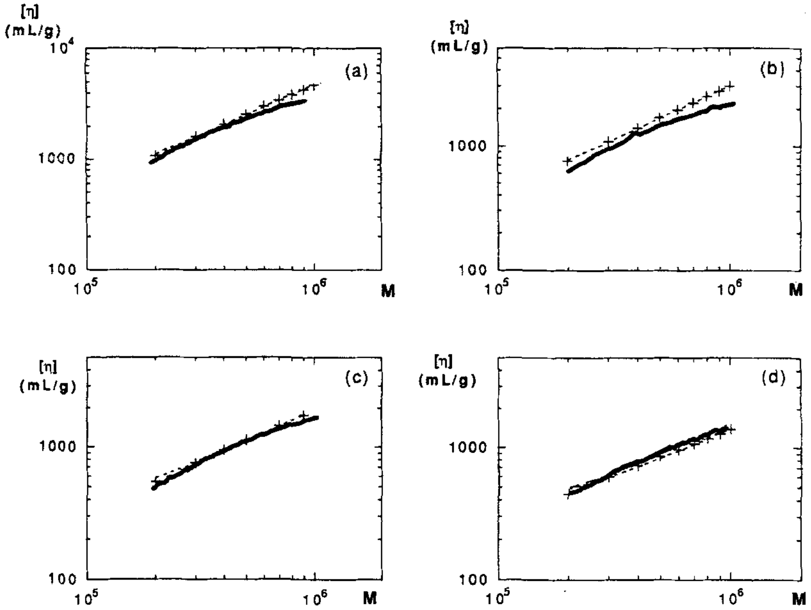

FIGURE 3 Log–log plot of the intrinsic viscosity [η] (mL/g) versus the molecular weight for different salt concentrations in which the experimental data (solid lines) are compared to theoretical values (crosses) obtained by setting $L_p = 60$ Å. (a) NH_4NO_3 10^{-2} M; (b) NH_4NO_3 3×10^{-2} M; (c) NH_4NO_3 10^{-1} M; (d) NH_4NO_3 3×10^{-1} M.

Influence of c_s on the Intrinsic Viscosity

SEC used in this study allows the determination of the reduced viscosity as a function of the molecular weight Figure 3. Correction for the finite concentrations was considered, but taking into account the low effective concentration in the SEC experiment (c_{max} corresponding to the maximum of the refractive signal is approximately 2.5×10^{-5} g/mL), it was proved to be negligible. As an example, one can write

$$\frac{\eta_{sp}}{c} = [\eta](1 + k'[\eta]c). \qquad (5)$$

From Figure 3, [η] is always greater than 400 mL/g and the maximum value of k' is 2 for $c_s \geq 10^{-2}$ M as determined separately.[8] As a consequence $k'[\eta]c \cong 0.02 \ll 1$. The direct determination of the reduced viscosity is a very good approximation of the intrinsic viscosity.

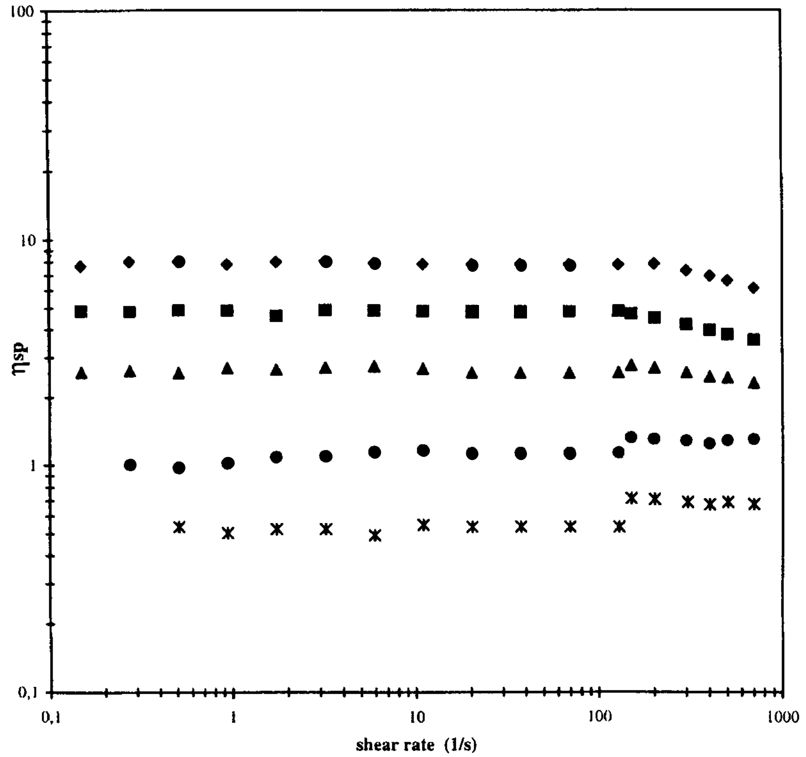

FIGURE 4 Variation of the specific viscosity with the shear rate for dilute solutions. HA sample: $M_w = 2.53 \times 10^6$ and $[\eta] = 3150\,mL/g$ in 0.1 M NaCl. $\dot{\gamma} < 10^2\,s^{-1}$ with LS 30; $\dot{\gamma} > 10^2\,s^{-1}$ with Carri-med. (✱) 0.128 g/L; (●) 0.256 g/L; (▲) 0.51 g/L; (■) 0.765 g/L; (◆) 1.02 g/L.

For moderate molecular weight values investigated and at low polymer concentration, it was previously shown that no shear-rate effect up to $\dot{\gamma} = 10^3\,s^{-1}$ was expected in 0.1 M NaCl. An example obtained in static viscosity measurements is given in Figure 4.[3,13] Thus, all the experimental values are expressed as $[\eta]$ without correction for finite concentration and shear-rate effect.

The experimental values were compared with calculated ones assuming a value of L_p that gave the best agreement. The value found was $L_p = 60\,Å$, which is a lower value than that introduced for the interpretation of $\langle\rho^2\rangle(M)$; this type of discrepancy also was found previously for hyaluronan.[3] The effect of shear rate may explain the curvature observed for experimental values in the range of highest molecular weights and lowest ionic strengths.

The slopes of the theoretical predictions given in Figure 3 in the range of molecular weights covered are the Mark–Houwink exponents *a* given in Table I; the values were calculated following the model developed previously.[14]

Steric Exclusion Chromatography Mechanism

Our objective was to investigate the validity of universal calibration for polyelectrolytes. Previously, it has been demonstrated that the same calibration was obtained for neutral polymers and polyelectrolytes in excess salt,[15,16] that is, when the Debye length becomes negligible compared to the radius of gyration of the polymer. The salt introduced in the eluent has an important role to screen electrostatic exclusion from the porous support and from interchain interactions.

In Figure 5 universal calibration is plotted, that is, the logarithm of the hydrodynamic volume $[\eta]M$ as a function of elution volume. All the experimental points in the range of fractionation give, to a good

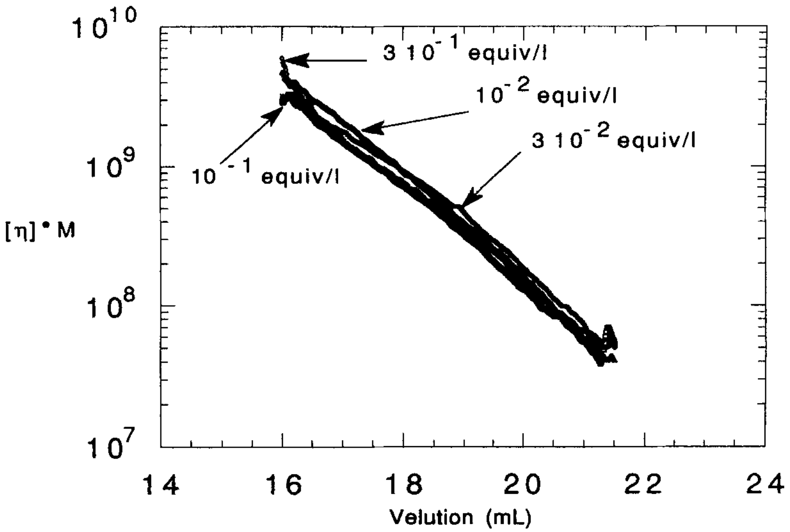

FIGURE 5 Universal calibration $[\eta]M(V_e)$ obtained for HA with different eluent salt concentrations.

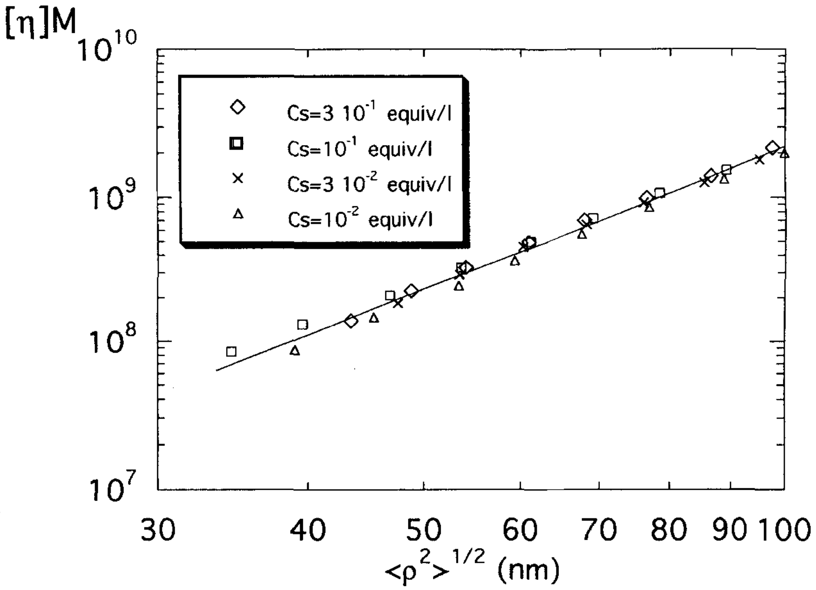

FIGURE 6 Variation of the hydrodynamic volume of HA as a function of the radius of gyration for the different ionic concentrations used for elution. M, $[\eta]$ and $\langle\rho^2\rangle^{1/2}$ are from independent measurements.

approximation, a unique curve within the limit of precision when $c_s \geq 10^{-2}$M. This result is in contradiction with the recent data described by Reed[17] who claimed that universal calibration failed with HA and other polyelectrolytes when eluted with different salts. Figure 6 is a plot of the hydrodynamic volume $[\eta]M$ as a function of the radius of gyration; all the values are obtained independently by viscometry and light scattering using SEC detectors. It is shown that $[\eta]M \propto R_g^{3.4}$; the slope 3.4 is not far from 3, as predicted.

For low salt content, that is, $c_s \leq 3 \times 10^{-3}$M, a large deviation was observed for $[\eta]M(V_e)$ (not shown), which seems to be a limit for the application of universal calibration; under these conditions, the Huggins constant k' is greater than 8 in the case of HA and $A_2 >$ 1.95×10^{-2} ($\text{mol} \cdot \text{cm}^3 \cdot \text{g}^{-2}$) which indicates significant long-range electrostatic repulsion. This effect especially exists when the electrostatic contribution to the Huggins constant is high and when a peak is observed when the reduced viscosity is plotted as a function of polymer concentration, as discussed previously.[8,18]

CONCLUSION

The results described in this paper correspond to an application of the wormlike chain treatment for polyelectrolytes. The combination of SEC with multidetectors has the advantage of fractionating the polymer and following the molecular weight during characterization. From these data, the influence of the ionic strength on the dimensions of ionic water-soluble polymers is directly obtained and analyzed in terms of a theoretical approach. In addition, it is confirmed that universal calibration for SEC is valid for ionic polymers as long as the ionic concentration of the eluent is equal or higher than 10^{-2}M for 1–1 electrolytes.

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