Influence of ionic strength on the flexibility of alginate studied by size exclusion chromatography

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Abstract

SEC measurements of the [η]-M relationship for alginate from *M. pyrifera* and the wormlike model can be used to characterize flexibility through two independent treatments (Bohdanecky's equations and HYDFIT program), both providing the same results. Two different assumptions concerning mass per unit of length lead to different conclusions. First: persistence length decreases with ionic strength (the intrinsic component of the persistence length is 11.3 nm and the electrostatic component is 6 nm when ionic strength is 0.01). Second: persistence length is independent of ionic strength (12 nm). Either of these options shows that the wormlike model in itself is not sufficient to explain flexibility over the whole range of chain lengths for these polyelectrolytes. A plausible explanation could be the presence of a combination of short-range and long-range screening effects of the ions of the solutions. This would also explain some data found in the literature regarding alginate flexibility.

1. Introduction

Alginates are structural biopolymers which comprise a broad family of polysaccharides found in brown seaweeds (*Laminaria sp., Macrocystis sp., Lessonia sp.* and others) (Smidsrød & Skjak-Bræk, 1990**)**, from which they are produced industrially. In addition, bacterial (*Azotobacter vinelandii, Pseudomonas aeruginosa*, and others) biosynthesis may provide alginates with more defined chemical structures and physical properties or may even enable production of alginate with tailor-made features (Remminghorst & Rehm, 2006). Their natural, rich and renewable sources and non-toxic characteristics, accompanied by the ability of alginates to form soft hydrogels in the presence of calcium ions, form the basis for a wide variety of applications in the food industry, pharmacy, agriculture and environmental science (Paul, 2008). Their versatility and biocompatibility provide an explanation for the wide interest shown in these molecules. For example, as biomaterials, alginates can easily be formulated into a variety of soft, elastic gels, fibers, foams, nanoparticles, multilayers etc. in physiological conditions that ensure the preservation of cell viability and function (Andersen, Strand, Formo, Alsberg & Christensen, 2012; Goh, Heng & Chan, 2012). In addition, it has been found that the alginate secreted by Pseudonomas aeroginosa in the bronchial tract contributes to many of the problems encountered in Cystic Fibrosis (Morris & Harding, 2009).

From a chemical point of view, alginate is a heteropolymeric, strictly linear (unbranched) chain which consists of β-1,4-linked mannuronic acid (or its salt form) residues (M) and its C5-epimer, α-L-guluronic acid (or its salt form) (G) (see, for example, Draget, Smidsrød & Skjäk-Braek, 2005). Alginates isolated from algae are generally of high molecular weight, typically in the range of 10^5 to 10^6 Da, corresponding to about 500-5000 residues per chain. Some bacterial alginates may be even larger (Steigedal et al., 2008).

The properties of dilute alginate solutions reflect the flexibility and extension of the chains, which

are usually considered to be semiflexible. Two characteristics of these polysaccharides are essential to explain their flexibility: the sequential structure of the chain (amount and distribution of G and M monomers) and the polyelectrolytic nature of these molecules. This aspect can affect their conformational properties (Volk, Vollmer, Schmidt, Oppermann, & Huber, 2004) because intramolecular repulsion between negative charges (polyelectrolytes are macromolecules which can become ionised when they are dissolved in aqueous media) add to the expansion caused by ring and binding geometries. These charges along their skeleton provoke changes which can be tuned by the presence of inorganic salts. High ionic strength (I) leads to a decrease in the viscosity of the solution compared with salt-free conditions because when an inert salt is added the screening of the charges that takes place means that the electrostatic interactions decrease and the conformation of the chain becomes more compact (Pamies, Rodríguez Schmidt, López Martínez & García de la Torre, 2010). Despite this simplified picture, polyelectrolytes in solution are considered among the least understood systems, especially compared with neutral polymer solutions (Yethiraj, 2009).

Data sets of radius of gyration (R_g), intrinsic viscosity ([η]), sedimentation velocity (s) and even the translational diffusion coefficient (D) versus molecular weight permit not only simple estimates of chain conformation type (sphere rod, coil, etc.) from a power law ($P=KM^{ap}$, with $P=R_G$, [η], s, D) or ‘‘Mark–Houwink–Kuhn–Sakurada’’ (MHS)-type analysis but also estimates of the flexibility via the chain persistence length (L_p) from more sophisticated representations. But, a survey of the literature shows that the determination of persistence lengths for polysaccharides in general (Morris et al. 2008) and for alginates in particular (Vold, Kristiansen & Christensen 2006) is not trivial because of both, experimental and theoretical-modelling-data processing difficulties.

Nowadays size exclusion chromatography (SEC) is probably the most widely used method for the molecular characterization of polymers in general. SEC has the advantage that the sample is chromatographically separated according to the molecular size (actually according to the

hydrodynamic volume, which differs markedly for different macromolecular architectures), and the chromatogram gives an immediate impression of the size distribution (broad/narrow, mono- or multimodal) (Mori & Barth, 1999). A breakthrough in SEC was the development of online multidetectors, especially light-scattering detectors, viscometers and mass spectrometers detectors (Gaborieau & Castignolles, 2011). Such SEC-multidetector systems provide the concentration profiles and molecular weights, radii of gyration and intrinsic viscosity for each elution slice.

Ortega and García de la Torre (2007) developed a new combined analysis method, which forms the basis of the software package, Multi-HYDFIT (Ortega & García de la Torre, 2013), a new global method combining all available data sets and minimising a target (error) function, that permits a more robust analysis. Using a combination of the Bohdanecky (1983) and Yamakawa and Fujii (1973) representations of worm-like coils, this program gives a combined or ‘‘global’’ estimate of the worm-like chain parameters: L_p and also the M_L (mass per unit length) and d (the chain diameter).

We present a study of the influence of ionic strength on the flexibility of alginate using the SEC technique. With this source of experimental data, two different data treatments are used to obtain L_p, including the HYDFIT analysis program which seems to be the best choice at present.

2. Materials and Methods.

2.1. Materials.

Sodium alginate was acquired from Sigma–Aldrich (referenced as A2158). These polymers are extracted from the alga *Macrocystis pyrifera* and have an M/G ratio of 1.56 (M=61%, G=39%). Dilute solutions of sodium alginate were prepared by dispersing the polymer in a previously

prepared $NaNO_3$ solution of the proper concentration for the desired ionic strength and stirring for one day at room temperature. All solutions were filtered (0.22 μm filters) to remove impurities before use. For this type of alginates dn/dc=0.150 (Martinsen, Skjåk-Bræk, Smidsrød, Zanetti, & Paoletti, 1991).

2.2 Capillary viscosimetry.

We determined the intrinsic viscosity, [η], by the traditional technique of capillary viscometry. Solutions were prepared by isotonic dilution (Morris, Cutler, Ross-Murphy, Rees & Priece, 1981) and measured using Ubbelhode viscometers (model AVS 310 from Schott Geräte). The size of the capillaries was 0.53 and 0.63 mm, depending on the flow time of the solution. All the experiments were carried out at 303 K and the solution flow times were in the range of 150-400 s. The intrinsic viscosity was calculated by double extrapolation to a zero concentration using the classical equations of Huggins (1942) and Kraemer (1938). The data were analyzed by means of VISFIT, a computer program developed in our research group and available in our webpage http://leonardo.fcu.um.es/macromol. This program simultaneously fits the data to both equations providing a unique result of [η], as detailed in López Martínez, Díaz, Ortega & García de la Torre (2003).

2.3. SEC with Online Multiangle Laser light-scattering and Viscometry.

Measurements were carried out at 303 K. The system consisted of a solvent reservoir, on-line degasser, pump, autoinjector, precolumn, and three columns (serially connected): an A6000M and A4000 both from Viscotek and a PL-aquagel-OH-40-8 μm from Agilent Technologies. In some experiments, the first column was changed for a TSK gel G5000 PWXL from Tosoh Bioscience.

The column outlet was connected to a triple detector array (TDA305 from Viscotek), to measure refraction index, light scattering (two angles RALS at 90º and LALS at 7º) and viscosity (4 capillary differential Wheatstone bridge configuration). The integrated detectors and columns were fully temperature controlled. The flow rate was 0.5 mL/min in all cases. The injection volume was 100 µL, and the sample concentration was adjusted to 4mg/ml for best results in the [η]-M relationship data. $NaNO_3$ solutions were used as mobile phases.

System, data acquisition and analysis were handled by OmniSEC sofware (Viscotek). This software calculates M and [η] independently and with no modeling assumptions from signals from light scattering detectors and viscometer. To obtain M we have assumed the approximation $2A_2Mc \approx 0$, being A_2 the second virial coefficient. This software also calculates R_g using the random coil model and we found that these experimental conditions were not suitable to obtain reliable data for R_g below $M=10^5$ Da. As a consequence we have not used R_g data in this work. OmniSEC calculates R_h using the well known Einstein's equations assuming the molecule is a hard sphere. This is not a valid assumption for our system, and in this work R_h values are only used to have an idea of the evolution of the relative size of the molecules with ionic strength and no conclusions are extracted from this parameter.

Data from slices were exported to a spreadsheet for further processing. In order to avoid uncertainties, the data for the very low or very high M slices were not included in the analysis.

2.4. Data treatments.

We used two different treatments to obtain L_p, M_L and d from the experimental data. First, we analyzed the [η]-*M* data by basically following the analysis performed by Mendichi, Soltés, & Schieroni (2003) on hyaluronan, based on the equations introduced by Bohdanecky (1983) in which

166 $(M^2/[\eta])^{1/3}$ is a linear function of $M^{1/2}$. The same procedure was used by Vold, Kristiansen &
167 Christensen (2006) to quantify the flexibility of alginates obtaining persistence length from SEC
168 data.
169
170 Secondly, the HYDFIT analysis introduced by Ortega and Garcia de la Torre (2007, 2013) was used
171 to estimate the molecular structure or parameters by performing a global weighted fit of multiple
172 samples in order to minimize a target function. The procedure was recently upgraded to include
173 more advanced computational procedures for predicting the properties of wormlike chains (Amorós,
174 Ortega & García de la Torre, 2011), and allows the simple, efficient and accurate estimation of the
175 wormlike chain parameters L_p, M_L and d. In order to reduce the number of parameters fitted
176 simultaneously, an estimate for the chain diameter *d* can be fixed a priori because the minimization
177 procedure is not generally sensitive to the value chosen (Patel et al., 2008). Furthermore, in cases
178 where M_L is known, the minimization procedure may yield a better defined value for L_p. In the
179 present work the hydrodynamic radius was obtained from intrinsic viscosity.
180
181 **3. Results and discussion**
182
183 **3.1. Global SEC results.**
184
185 We studied a polydisperse (M_w/M_n=2.32) relatively small (M_w=107 KDa) sample of alginate from
186 *M. pyrifera* (Table 1). The global values of [η] (obtained from capillary viscosimetry and SEC) and
187 the hydrodynamic radius show a relatively small decrease in dimensions and intrinsic viscosity as
188 the ionic strength increases.
189
190 The agreement between M_n and M_w obtained at different ionic strength is good. Only values for
191 I=0.4 clearly disagree. The tendency of alginates to aggregate (or form microgels) when ionic

strength increases is well known (Mackie, Noy & Sellen, 1980). Figure 1 shows the weight fraction distribution for three ionic strengths. At I=0.01 and I=0.1 the weight distribution is similar, and only a nearly negligible displacement towards a higher M is observable. At I=0.4 the distribution is clearly broader, reflecting the presence of aggregates, which would explain the higher M_w than obtained in the other experiments.

Table 1.- Global data for alginate samples at different ionic strengths[a]

Ionic strength	0.01	0.05	0.1	0.2	0.3	0.4	average[b]
M_n (D) x10^{-3}	44 ±4	46 ±5	47 ±3	43 ±9	48 ±5	49 ±4	46 ±2
M_w (D) x10^{-3}	104 ±4	105 ±5	108 ±9	105 ±7	114 ±4	289 ±40	107 ±4
Radius - (nm)[c]	19.9 ±0.3	18.5 ±0.5	18.2 ±0.4	17.8 ±0.6	18.2 ±0.3	19.7 ±0.6	
[η] (cm^3/g)	601 ±1	474 ±2	453 ±12	427 ±8	418 ±1	405 ±9	
[η] (cm^3/g)[d]	657	480	464	432	--	390	

[a] Values and standard deviations for each ionic strength are obtained from data of 6 to 8 different injections of 2 different preparations.
[b] Averages and standard deviations from values of M_n and M_w at different ionic strengths with values for I=0.4 not included.
[c] Weight average.
[d] From capillary viscosimetry.

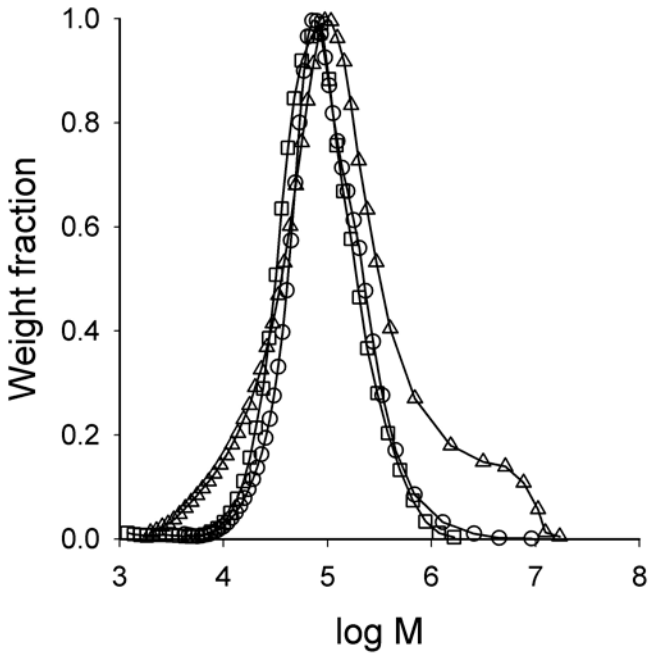

Figure 1.- Weight fraction distribution of one injection for I=0.01 (□), I=0.1 (○) and I=0.4 (Δ). For better comparison, each curve has been normalized to their maximum value.

To obtain M of each slice, we have assumed $2A_2Mc \approx 0$. A_2, the second virial coefficient, could depend on ionic strength (the lower I the higher A_2) and also on molecular weight. In practice, although a previous determination of A_2 should be desirable, the knowledge of A_2 in the whole range of I and in the whole range of M is very difficult. For reference a value of $B=29.0 \times 10^{-4}$ ml.mol/g^2 was given by Wedlock et al. (2006) for a polydisperse sample of M_w=350 kDa from *Laminaria hyperborean* at I=0.3. In our case, M_w=107 kDa. Although initial concentration introduced in the column is 4mg/ml, the actual concentration of the slices is much slower reaching a maximum of 0.1 mg/ml. With these data, we could estimate, on average, an underestimation around 5% for I=0.3. According with a plot shown by Horton et al. (1991) for alginate from *Laminaria hyperborean* at I=0.3 and with M_w=240 kDa, the underestimation in our case could be even lower. The worst conditions for our approximation to be valid could be when I=0.01. Table I shows that values for M_w are independent of ionic strength in our work, except for I=0.4. In addition, Figure 1 shows that the molecular weight distribution for I=0.01 and I=0.1 are nearly identical. Figure 1 also explains the main difference in M_w for I=0.4, showing that a significant number of aggregates have appeared, probably due to the high ionic strength. Finally, Table I shows that values for IV obtained from capillary viscosimetry are very similar to those obtained from SEC, what is re-assuring about the use of this approximation. Anyway, this approximation must be treated carefully, because it could be a limiting factor, mainly when we work with longer chains and at low ionic strengths.

3.2. Results for infinite ionic strengths.

To obtain results for infinite ionic strengths it is necessary to find a reliable extrapolation method using the Pal-Hermans equation (Pals & Hermans, 1950, 1952)

$$[\eta] = [\eta]_\infty + SI^{-1/2}$$

To handle the large amount of experimental data, a series of steps were followed. The first one involved fitting data to an appropriate curve. The MHS power law relationships between M and [η]

is usually accepted, but, according to our data, in a range from logM=4.5 to logM=5.8 there is a curved instead of a linear dependence (as expected from MHS law). This curvature was already observed by Vold et al. (2006) although their option was to divide the data into three ranges of molecular weights (Vold, Kristiansen & Christensen, 2007). We decided to fit our data to a second order polynomial equation, with good results (not shown). An illustrative example is shown in Figure 2 for I=0.1, including the fitting equation $\log [\eta] = -13.027 + 4.502 \log M - 0.35549 (\log M)^2$.

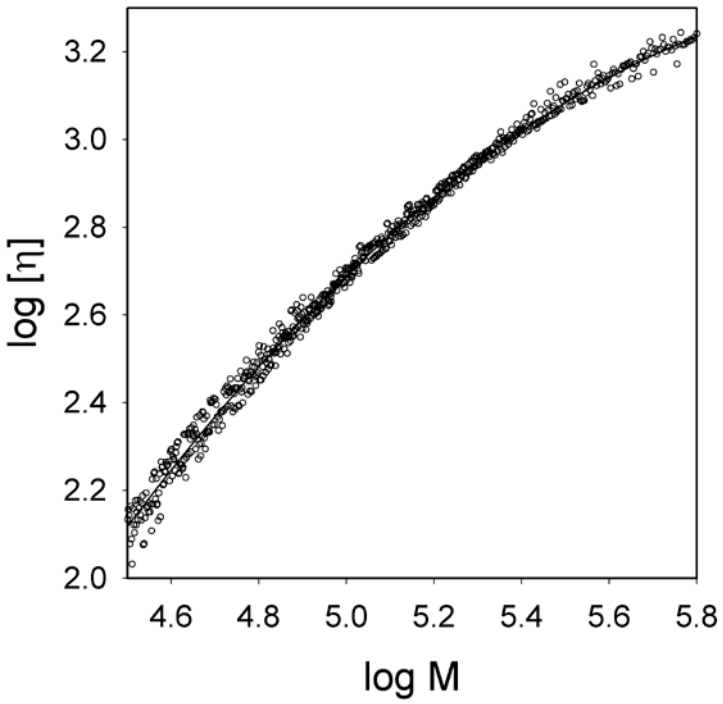

Figure 2.- Illustration of the fitting the experimental data to a second order polynomial equation in the log[η] versus log M plot. The data shown are for I=0.1. The fitting equation is: $\log [\eta] = -13.027 + 4.502 \log M - 0.35549 (\log M)^2$. Two different alginate solutions were prepared with 8 injections for each. For better visualization, only 1 of every 75 data is shown in the plot.

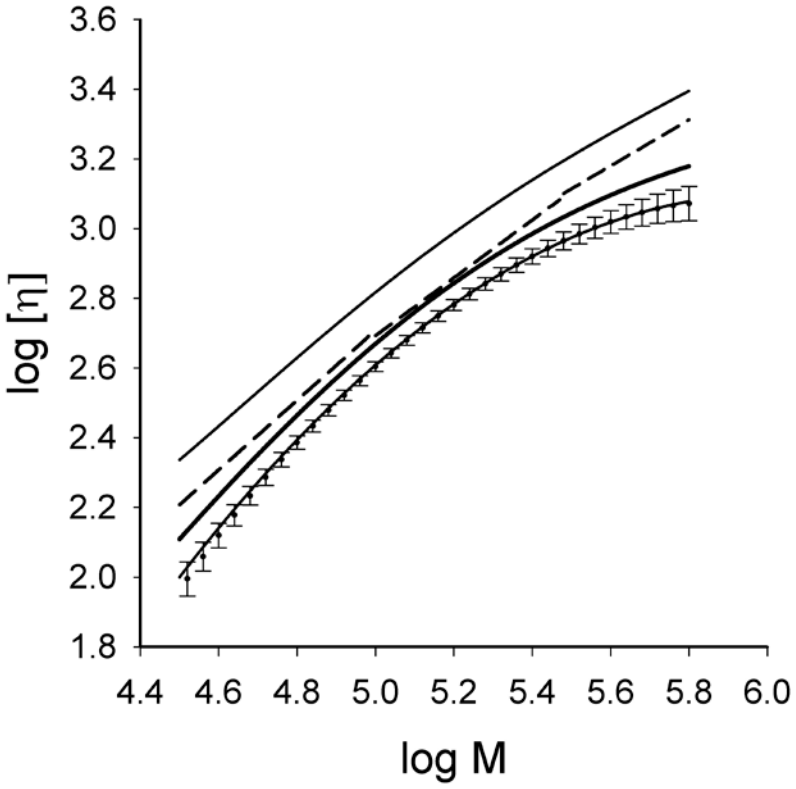

Figure 3.- Log plot of the [η]-M relationship for various ionic strengths obtained after taking into account all experimental results (solid lines). From lower to higher: I=∞, I=0.17 and I=0.01. $[\eta]_\infty$ was obtained as the average of all ionic strengths according to equation 4. Error bars are included. $[\eta]_{0.01}$ and $[\eta]_{0.17}$ were obtained from $[\eta]_\infty$ and equation 4. Results from Vold et al. (2007) for I=0.17 are included for comparison (dashed line). [η] is in mL/g; M in Da.

The second step was to obtain S as a function of M. We observed that a second order polynomial described the S dependence on M very well ($S = 2.351 + 2.333x10^{-4} M - 6.244x10^{-11} M^2$). Once this dependence is known $[\eta]_\infty$ could be readily obtained for each value of M by applying the Pal-Hermans equation from data of any ionic strength. We decided to take into account all available data and we obtained the averaged value of $[\eta]_\infty$ for each M. Later, data were fitted to a second order polynomial equation as shown in Figure 3.

Another option, which was used by Vold. et al. (2006, 2007), would be to use the empirical B parameter (Smidsrød & Haug, 1971), assuming that is molecular weight-independent. B can be

obtained from the Pals-Hersman equation defining $S = B[\eta]_{0.1}^{v}$, with v=1.3 (Dentini, Rinaldi, Risica, Barbetta & Skjåk-Bræk, 2005). We obtained B from the SEC quasi-monodisperse fractions and since our results showed a significant dependence on M this option was discarded.

3.3. Obtaining persistence length.

We applied the HYDFIT analysis and Bohdanecky's equations to the M and $[\eta]$ data sets obtained from SEC elution slices to obtain persistence length of alginate molecules.

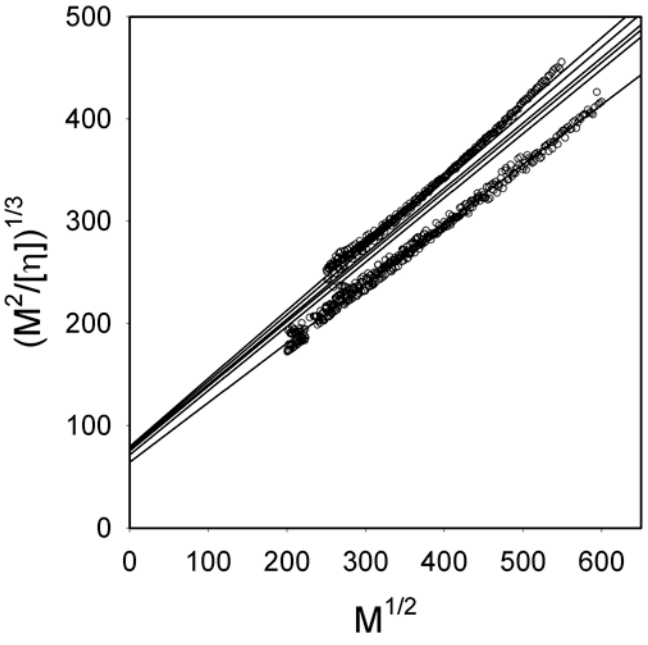

Figure 4.- SEC results in the form of Bohdanecky's equations. Only some experimental data for two values of I (I=0.01, lower in plot and I=0.4, higher in plot) are shown for clarity. For the same reason, only 1 of every 50 experimental points is plotted. Lines are obtained from linear regression in slightly different molecular weight ranges (see text) and correspond, from lower to higher, to I=0.01, 0.05, 0.1, 0.2, 0.3 and 0.4. Fitting results are shown in Table 2.

When using Bohdanecky's equations (Figure 4), linear regressions of $(M^2/[\eta])^{1/3}$ versus $M^{1/2}$ for each ionic strength give the intercepts and slopes and therefore L_p, which is sensitive to slight

differences in these parameters. In the data fitting procedure it is desirable to use the widest possible range of molecular weights, although we found that the range of linear behavior decreased significantly with increasing I. To increase the consistency of our data between 8 and 15 different injections of two different preparations were used. In addition we used two different (although similar) sets of chromatographic columns. Accordingly, ranges run from M=40000 to 360000 for I=0.01 to M=60000 to 300000 for I=0.4. Values for the fitting parameter R^2 are always over 0.99, what it is reasonably taking into account that the actual number of data for each fitting is several thousands. Providing the partial hydrodynamic volume ($\bar{\upsilon}$) is known, the hydrodynamic diameter can be calculated and then M_L and L_p. We used $\bar{\upsilon}$=0.54±0.06 mL/g (Martin, Cook & Winkler, 1956). Values for M_L and L_p are shown in Table 2.

According to this treatment, the persistence length is around 12.0 nm and does not change significantly with the ionic strength of the solution (at least in the range we used) and the changes are produced in M_L (from 323 to 420 nm^{-1}) and, to a less significant extent, in the diameter of the chain.

Table 2.- Values for M_L and L_p for different ionic strengths obtained from Bohdanecky's equations and the HYDFIT procedure.

	HYDFIT				Bohdanecky		
I	d (nm)	M_L (Da/nm)	L_p (nm)	Δ^2 (x10^2)	d (nm)	M_L (Da/nm)	L_p (nm)
0.01	0.64	321	11.7	2.13	0.61	323	12.1
0.05	0.60	384	13.2	2.50	0.65	365	11.8
0.1	0.66	383	12.1	1.79	0.66	384	12.2
0.2	0.66	383	11.5	1.42	0.67	391	12.2
0.3	0.66	420	13.1	2.30	0.68	401	11.9
0.4	0.80	413	11.3	1.23	0.69	412	11.8
∞	0.71	464	14.2	1.26	0.70	426	12.0
Average			12±1				12.0±0.2
0.01			19.3	3.21			15.5
0.05			14.7	2.45			13.2

0.1	0.69	410	13.7	1.81	0.69	410	13.1
0.2			13.2	1.51			12.8
0.3			12.1	2.45			12.2
0.4			11.6	1.36			11.8
∞			11.1	1.35			11.5

From data with M in g/mol and [η] in cm^3/g

The M_L value can be obtained theoretically from the chemical structure, according to the relation

$$M_L = \frac{F_G M_{0,G} + (1 - F_G) M_{0,M}}{F_G b_G + (1 - F_G) b_M}$$

Where F_G is the fraction of L-guluronic acid residues, and $M_{0,G}$ and $M_{0,M}$ are the molar masses of the two monomers (both 198 g/mol for the Na^+ salt). The bond distances are 0.515 nm (b_M) and 0.435 nm (b_G), respectively, with the first referring to the M in the $^4C_1$ form, and G in the $^1C_4$ form (Smidsrød, Glover & Whittington, 1973). In our case, M_L=410 Da/nm, and with this constant value, L_p values also can be obtained as shown in Table 2.

The use of HYDFIT is simpler because it is simply necessary to introduce a reasonably wide range of d, M_L and L_p. After introducing the same sets of input data as used with Bohdanecky's equations we obtained the results shown in Tables 2 after optimizing a target function Δ (Figure 5).

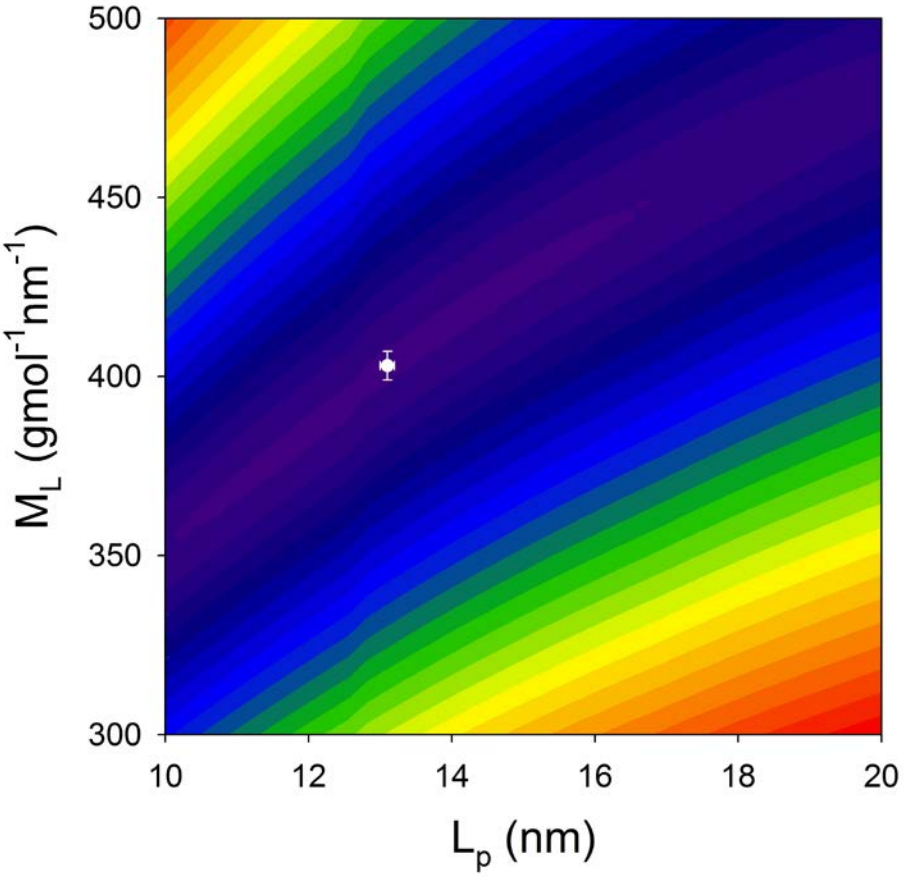

Figure 5.- Contour plot for HYDFIT target function Δ for I=0.1 with d=0.69 nm. In this representation the values of Δ are presented by the full colour spectrum, from blue (Δ=0.05) to red (Δ=25). The minima (with error bars is also plotted (white).

3.5. Flexibility of alginate

We have used the wormlike model to quantify the flexibility of alginate molecules. Both data treatments (Bohdanecky's equations and the HYDFIT program) give very similar results for persistence length (Tables 2). However, it must be concluded that this model is not sufficient to describe the [η]-M relationship over a wide range of M (see Figure 4 as an illustration), an observation that becomes relevant as I increases. Vold et al. (2006) also found that the wormlike chain may not be the most suitable model when the flexibility of alginate molecules increases, as occurred in their work in the absence of excluded volume effects for the most oxidized alginate samples.

In the analysis of our experimental data two different assumptions led to different conclusions. First, as frequently done in previous studies, M_L was assumed to be constant. The expected increase in persistence length as I decreased was indeed observed. For polyelectrolytes, persistence length has usually been described as the sum of two contributions (Odijk, 1977; Skolnick & Fixman, 1977): the intrinsic contribution, due to the chemical structure of the chain in the absence of intramolecular electrostatic interactions, and the electrostatic contribution due to the polyelectrolytic nature of the molecule. In our case, the L_p values of 11.5 and 11.1 nm were obtained for $I=\infty$ (averaged, 11.3 nm). The value of this intrinsic contribution is only slightly smaller than that (12.5 nm) obtained by Zhang H., Wang H., Wang J., Guo R., Zhang Q. (2001) for longer alginates from *L. nigresens* following the method suggested by Odijk (1977). This value is also close to that (12 nm) proposed in the more recent paper of Vold et al. (2006) for *L. hyperborean*. We find that the electrostatic contribution to persistence length at I=0.01 would be around 6 nm (see Table 2). This value is higher than the one obtained by Zhang et al. (2001) (3.2 nm). At I=0.2, we find the electrostatic contribution to Lp to be 1.7 nm, while, for I=0.17, Vold et al. (2006) obtained 3.3±0.3 nm (M_L = 440±10 Da/nm) and 2.7 nm (M_L=424 Da/nm). Although with some differences, all these values mean that the intrinsic contribution to flexibility of the structure of the chain would be higher than the effect of the external salt in this range of ionic strengths.

Another possibility was not to impose any restriction for calculating M_L or L_p (indeed, with the large set of M-dependent [η], both parameters can be safely adjusted simultaneously with the Bohdanecky method or the HYDFIT program). In this case (see Table 2) a new explanation (we have not found it in the literature) of the effect of the presence of inorganic ions in solutions of alginates (and perhaps of other polyelectrolytes) can be deduced: the increase in ionic strength would produce a decrease in contour length and, as a consequence, in M_L (also a small change in diameter) and small changes in L_p which value would be around 12 nm.

HYDFIT allows Δ to be obtained, that is, a parameter to evaluate the goodness of the found target function. This parameter is always slightly smaller (better) for the treatment in which M_L is not fixed as opposed to when M_L is taken to be 410 Da/nm, which supports the second option.

A different and complementary approach is the analysis of the MHS exponent obtained from experimental data. All our SEC results show that an increase in I leads to a decrease in $[\eta]$ for molecules of the same M. In addition, once the $[\eta]_\infty$-M relationship was obtained taking into account all available data, it is a straightforward task to calculate the [η]-M relationship for any ionic strength (Figure 3). Such relationships would be valid in the I=0.01 to ∞ and logM=4.5 to 5.8 ranges.

Moreover, these relationships are well described by a second order polynomial, and, as a consequence, if $\log[\eta]=a_0+a_1(\log M)+a_2(\log M)^2$, a local MHS exponent dependent on M can be defined as $a_{[\eta]}(M)=a_1+2a_2(\log M)$ (see Figure 6).

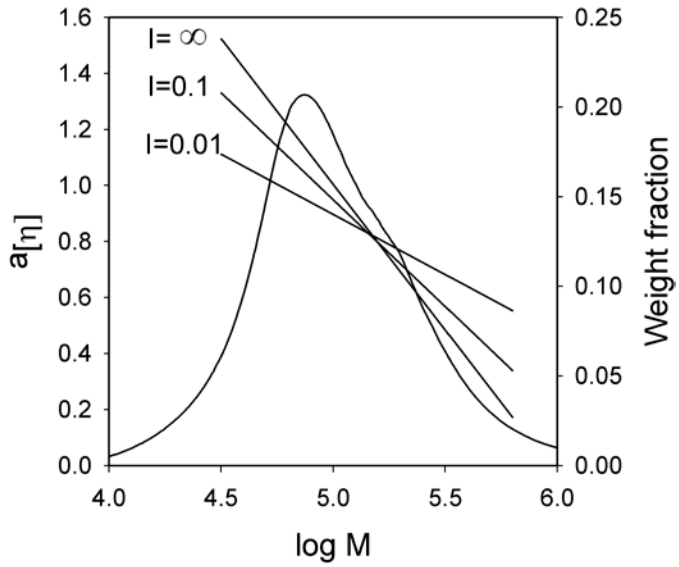

Figure 6.-Local Mark-Howking-Sakurada $a_{[\eta]}$ as a function of M for three different ionic strengths (straight lines). Weight fraction distribution of one sample (I=0.1) is also included as a reference (curve). Equations are as follows: I=∞, $a_{[\eta]}$= 6.204 – 1.040 log M; I=0.1, $a_{[\eta]}$ = 4.759 – 0.762 log M; I=0.01, $a_{[\eta]}$ = 3.047 –

0.430 log M.

Vold et al. (2006) found no qualitative differences in the dependence of M and [η] between I=0.17 and I=1. Our results (Figure 6) point to a range of M in which no or very slight differences in $a_{[\eta]}$ are observed for different values of I, even if significantly different flexibilities (or conformations) are observed at higher M. This might explain the results of the mentioned paper.

$a_{[\eta]}$ is usually used as a semi-quantitative measure of the rigidity or the expansion of the molecule (Harding, Abdelhameed & Morris, 2011), assigning more extended conformations to higher values of $a_{[\eta]}$. Therefore, Figures 3 and 6 show that, for low M values, molecules become more rigid (more extended) when I increases. The same plots show that in the high M range molecules become more flexible (less extended) when I increases. This apparent contradiction suggests a complex influence of the inorganic ions present in the solution, including the tendency of alginate molecules to aggregate when the number of ions present in the solution grows.

A plausible explanation for all these observations could be as follows. When I is low, the alginate molecules are well described by the wormlike model, but when concentration of inorganic ions grows two different effects could appear. On the one hand, short range interactions or screening between COO^- groups of contiguous or very close G and M monomers could shorten the contour length of the molecules as the salt concentration increases, giving rise to shorter (lower [η]) and more rigid molecules. At the same time long range interactions or screening between charges more separated in the chain could allow more folded conformations, and, for higher ionic strengths, would also allow aggregation among different molecules. These two effects would therefore be opposite and in competition and will depend on ionic strength. For shorter molecules, the first factor would be more important while the second would increase strongly with chain length.

3.6. Differences found in the literature.

A survey of the literature finds variable results in the characterization of flexibility of alginates. Vold et al. (2006) attributed the differences to sample quality and purification, as well as data processing. They also mention that slightly different analysis conditions (temperature, ionic strength, shear rate) were used, although these parameters generally fell within ranges that would be expected to play only a minor, or even negligible, role. Another source of difficulties could be the chemical composition of alginates. Indeed, experimental and theoretical studies have concluded that polyguluronate blocks (GG) are stiffer than polymanuronate blocks (MM), while heteropolymeric blocks (MG) are the most flexible (Mackie, Noy, & Sellen, 1980; Smidsrød, 1970), although some recent studies have found no such dependence on the composition (Vold, Kristiansen & Christensen, 2006; Storz et al. 2009; Josef & Bianco-Pelet, 2012). Our results show that polydispersity could add some complexity to the interpretation of the experimental results.

Although we found that the MHS equation is not a good representation of the experimental results for wide ranges of molecular weights, historically this form it has been used. In the same way as Vold et al. (2006) did, the ranges of M can be fractionated to perform an approximate fitting to a straight line of the relationship log[η]-logM. For illustrative purposes, we divided our results, for I=0.1, in four different M ranges obtaining $a_{[\eta]}$=1.13 for M=40-80 kDa, $a_{[\eta]}$=0.91 for M=80-160 kDa, $a_{[\eta]}$=0.68 for M=160-320 kDa and $a_{[\eta]}$=0.47 for M=320-630 kDa. The second range (see Figure 1) covers a large fraction of the total mass of the sample and is where the maximum RI signal is found in our SEC measurements. It is also a region in which the signal to noise ratio for all detectors is good and the one that includes the value of M_w. The MHS representation in this range of molecular weights gives very similar results to those of Martinsen et al. (1991) for alginates from the same source. But, if molecules of lower M are included, $a_{[\eta]}$ would increase, while the contrary would occur if molecules of higher M were included.

For comparison, we have included in Figure 3 the results obtained by Vold et al. (2007). The coincidence between both works is very good in the range between 100 and 200 kDa. But significant quantitative differences appear at low and high M. These differences could be somehow coherent with the results shown in our Figure 6, which shows that differences in conformations (although this figure compares the same alginate at different ionic strengths) are going to be small in a molecular range around 150 kDa. We think that this is an interesting topic of study in the future because we have not found a definitive explanation. One reason to explain the differences could be the nature of the alginate sample because the composition in terms of monomers is rather different (60% M versus 30% M, approximately) but, as suggested by different authors (Vold, Kristiansen & Christensen, 2006; Storz et al. 2009; Josef & Bianco-Pelet, 2012), we think that this is unlikely. Another factor to explain the difference could be the procedure to obtain molecular weight. Vold et at. (2006, 2007) used a multiangle light scattering detector and data processing according to the Zimm formalism while we have used a two angle light scattering detector as explained before. But, as we have also discussed above, we think that it is unlikely that differences are due to this factor. In addition, our Figure 3 show the curves obtained after taking into account all the results that we have measured at different ionic strengths, being all of them coherent in absolute and relative terms.

According to our results the influence of the chain length distribution and the ionic strength on the [η] of a polydisperse sample is very important and quite complex. Indeed, as mentioned by Vold et al. (2006, 2007), the $a_{[\eta]}$ parameter of the MHS equation decreases significantly at increasing molecular weights even for molecules with the same L_p. Moreover, for high I, not only individual molecules, but also aggregates of different sizes could be present, which might make $a_{[\eta]}$ to be even smaller. Finally, we remind that when simultaneously analyzing a mixture of different chain lengths, as in a polydisperse system, only an average is observed. As a consequence, the analysis of the MHS equations found in the literature would need to take into account these factors.

As an additional factor to understand the differences found in the literature, we must remind that the determination of absolute values of M is strongly affected by dn/dc, and there are significant differences between the values presented in different works from 0.168 (Rinaudo, 2008) or 0.165 (Theisen, Johann, Deacon & Harding, 2000) to the lower value of 0.150 (Martinsen et al., 1991).

4- Conclusions

SEC experiments of polydisperse alginate from *M. pyrifera* are an important source of information, but the quality and statistical robustness of data for a wide range of molecular weight are essential to reach reliable conclusions.

According to our results, the MHS power law does not provide a good description of the [η]-M relationship for wide ranges of molecular weights of monodisperse fractions. This could explain some of the differences in the interpretation of the [η]-M data found in the literature.

The quantitative determination of the parameters for these polyelectrolytic chains using the wormlike model shows two different scenarios. The first one appears if we assume that M_L (and so contour length and diameter) do not change by the effect of I. In this case the "classical" flexibility mechanism of L_p decreasing with ionic strength would be adequate. The intrinsic component of the persistence length would be around 11.3 nm and the electrostatic one would be around 6 nm when I=0.01. These values are close to previous results from literature. In the second scenario, we do not impose any restriction on the calculation of M_L or L_p in the wormlike model. Then a new description of the effect of inorganic ions on alginates (and perhaps other polyelectrolytes) could be offered. In this case, the decrease in [η] with increasing ionic strength would be due to by a decrease in contour length and hence in M_L. Any changes in the value of L_p which, in our case, is ±12 nm

would be slight. Although further investigation is needed, this option offers a new way to understand the complex mechanism of flexibility in polyelectrolytes.

Both of the two former options use the wormlike model, but this in itself is not enough to explain flexibility over the whole range of chain lengths, particularly at higher values of ionic strengths. One plausible explanation could be the combination of short-range and long-range screening effects of inorganic ions in the solutions. These effects would allow the formation of aggregates when the salt concentration is high enough.

Finally, it should be emphasized that additional information on R_g and other hydrodynamic properties would be desirable. The use of previously fractionated samples should reduce some of the difficulties provoked by polydispersity. Also the computer simulation of adequate models would help to improve our knowledge of the flexibility mechanisms of polyelectrolytes in general and alginates in particular.

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