Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Polymers 59 (2005) 489–499
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Comparative studies on solution characteristics of mannuronan epimerized by C-5 epimerases

M. Dentini[a,*], G. Rinaldi[a], D. Risica[a], A. Barbetta[a], G. Skjåk-Bræk[b]

[a]Department of Chemistry, University of Rome 'La Sapienza', P.le A. Moro, 5, Rome 00185, Italy
[b]Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway

Received 25 May 2004; revised 7 October 2004; accepted 9 November 2004
Available online 15 December 2004

Abstract

Both dilute and concentrated solutions of bacterial Mannuronan (MANNA) and its epimerized products by AlgE1 at 5 and 24 h, named MANNAEp1t5h and MANNAEp1t24h, respectively, and AlgE4 (MANNAEp4) have been studied as a function of variables such as polymer concentration and ionic strength (NaCl) in order to investigate the macromolecular solution properties of these innovative polyuronic acids having the same charge density but different composition and sequence of β-D-mannuronic acid (-M-), α-L-guluronic acid (-G-) or MG-blocks.

Measurements of intrinsic viscosity $[\eta]$ as a function of ionic strength, I, by capillary viscometry has led to an estimate of the Smidsrød-Haug parameter B, an index useful to characterize the stiffness of polymeric chains. The results are largely consistent with much of the published data relative to chain extension and conformational freedom around the torsional angles of the glycosidic linkages occurring in alginates.

Steady shear rheometry provided information about the coil-overlapping parameter c^*, which marks the transition from dilute to concentrated solution. The slopes of the double logarithmic plots of η_{sp} vs. $c[\eta]$ both at low and high degrees of coil overlap suggest that all samples solutions behave like linear polymer entangled network systems. The value of c^* is strictly influenced by the stiffness of the chains, and hence by the primary structure.

Dynamic shear rheometry shows that the frequency dependence of dynamic viscosity is only partially superimposable to the shear rate dependence of viscosity. Such behaviour may be ascribed to the presence of semiflexible polymeric coils in a non-totally destructured entangled state.

By solvent/non-solvent (H_2O/isopropanol) fractionation carried out on mannuronan, a set of samples with different average molecular weights and narrow polydispersities were obtained. Triple detection GPC allowed the evalutation of the Mark–Houwink–Sakurada parameters as well as of the characteristic ratio C_∞ for one of the fractionated MANNA samples. The chain persistence length was estimated by the wormlike chain model.

Keywords: Alginate; Mannuronan C-5 epimerases; Viscometry; Rheology; Flexibility of polysaccharides

1. Introduction

Alginate is a family of polysaccharides composed of (1→4)-linked residues of β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G) arranged in varying block wise patterns along the linear chain.

It occurs as the major structural polysaccharide of brown seaweeds. Both the fraction and the sequence of the two residues depend upon the source from which the polymer is extracted (Ertesvåg & Valla, 1998).

The biotechnological, biomedical and pharmaceutical applications of alginates in solution and in the gel–phase are largely determined by the relative amounts of the three types of block present (M-blocks, G-blocks and MG-blocks). For instance, the entrapment of cells and enzymes is achieved

* Corresponding author. Tel.: +39 0649913633; fax: +39 06490631.
E-mail address: mariella.dentini@uniroma1.it (M. Dentini).

0144-8617/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2004.11.006

by exploiting the gelling properties of alginates rich in homoguluronic sequences in the presence of multivalent cations such as Ca^{++}, Sr^{++} and Ba^{++} (Braccini & Pérez, 2001; Stokke, Smidsrød, Bruheim & Skjåk-Bræk, 1991). The strength of alginate gels, their stability and biological effect on the entrapped system strongly depends on the composition of the polymer. MG-sequences have been demonstrated to have an inhibitory effect on pepsine, while alginates rich in polyM sequences have a strong cytokine-inducing ability on human monocytes (Skjåk-Bræk & Espevik, 1996 and references therein).

The biosynthesis of alginates proceeds through the polymerization of fructose, followed by a post-polymerization C-5-epimerization of β-D-ManA residues to its C-5 epimer, α-L-GulA. Various recombinantly produced mannuronan C-5 epimerases based on genes from *Azotobacter vinelandii* are now available, and it is reported that they introduce different sequence patterns in mannuronan and alginates with an initial low fraction of guluronic acid (Ertesvåg et al., 1995). These enzymes can therefore be used to modify alginates in vitro to obtain pseudoalginates with the desired content and distribution of G residues (Draget, Skjåk-Bræk, & Smidsrød, 1997).

AlgE1 is a M-5 epimerase endowed with a double epimerasic activity (Ertesvåg, Høydal, Skjåk-Bræk, & Valla 1998). Indeed, it turns the mannuronan substrate into a derivative characterised by long G-blocks and MG-sequences. It is of interest to verify if this dual activity can be somehow temporarily separated. AlgE4, on the other hand, is known to introduce alternating MG-sequences into the alginate chain (Hartmann, Holm, Johansen, Skjåk-Bræk, & Stokke, 2002).

Mannuronan, an uncommon alginate completely constituted by mannuronic residues produced in large quantities by an epimerase negative mutant of *P. fluorescens* (Gimmestad et al., 2003), is an excellent substrate to study the effect of increasing the amount of G-blocks and MG-blocks on the mechanical properties of the epimerised mannuronans solutions both in dilute and concentrated regimes. In this paper we aim at correlating the chemical composition to the solution properties of these innovative polyelectrolytes.

Furthermore MANNA samples with different molecular weights and narrow polydispersities were obtained using a solvent/non-solvent fractionation procedure. We report the results of the triple detection–GPC measurements carried out on the least polydispersed fraction. The Mark–Houwink–Sakurada parameters and characteristic ratio C_∞ were determined.

2. Experimental

2.1. Materials

High molecular weight mannuronan was produced from the fermentation of D-fructose-6-phosphate in the presence of epimerase-negative $AlgG^-$ strain of *P. fluorescens* PF20118, provided by SINTEF, Applied Chemistry (Trondheim, Norway). Pure mannuronan was epimerized in vitro by AlgE1 at different times (5 and 24 h) and by AlgE4. Purification and deacetylation were carried out as described earlier (Ertesvåg & Skjåk-Bræk, 1999). The mannuronan C-5 epimerases AlgE4 and AlgE1 were produced by fermentation of recombinant strains of *E. coli*, JM109 and JM 105, respectively (Høydal, Ertesvåg, Stokke, Skjåk-Bræk, & Valla, 1999; Ertesvåg et al., 1998). The enzymes were preliminary purified by ion exchange chromatography on Q-Sepharose FF (Pharmacia, Uppsala, Sweden) and by hydrophobic interaction chromatography on Phenyl Sepharose FF (Pharmacia). The relative activity of the enzymes was assayed through the determination of the amount of tritium released in water when the enzymes were incubated with ^{3}H-5 labelled mannuronan (Ertesvåg & Skjåk-Bræk 1999).

The fraction of guluronate (G) residues, the mole fraction of GG, MM and GM (MG) diad sequences F_{GG}, F_{MM} and F_{MG} were determined by ^{1}H-NMR (Grasdalen, 1983) in D_2O at 90 °C. The molecular weights were determined by size exclusion chromatography multi-angle laser light scattering (SEC-MALLS) (Donati et al., 2004). The characterization results are summarized in Table 1.

2.2. Intrinsic viscosity measurements

The intrinsic viscosity $[\eta]$ used for the calculation of B parameters, were determined using the Viscosity measuring unit AVS/G (Schott-Geräte), connected to a AVS/T100 Piston Buret (for automatic dilutions). This makes

Table 1
Chemical composition, sequence parameters and average ponderal molecular weight of mannuronan (MANNA), mannuronan epimerized by AlgE4 (MANNAEp4), mannuronan epimerized by AlgE1 for 5 h (MANNAEp1t5h) and mannuronan epimerized by AlgE1 for 24 h (MANNAEp1t24h)

Sample	F_G	F_M	F_{GG}	F_{MM}	F_{MG}	$N_{G>1}$	M_W
MANNA	–	1	–	1	–	–	496,000
MANNAEp4	0.47	0.53	–	0.06	0.47	–	533,000
MANNAEp1t5h	0.44	0.56	0.32	0.44	0.12	13	444,000
MANNAEp1t24h	0.68	0.32	0.59	0.23	0.09	22	452,000

F_G and F_M are the molecular fractions of guluronic and mannuronic acid, respectively. F_{GG}, F_{MM}, F_{MG} ($=F_{GM}$) are the diad frequencies, while $N_{G>1}$ is the average number of G-blocks.

automated measurements of the flow-through times in a capillary viscometer (Ubbelohde viscometer for dilution sequences). The viscometer was immersed in a precision water bath (transparent thermostat, CT 1150) to maintain the temperature at 30 ± 0.05 °C. All polymer concentrations ranged from 0.05 to 0.019 (w/v) so that the viscosity relative to that of the solvent (NaCl solution) lay in the range $1.2 < \eta_r < 2.0$.

To get the desired solution ionic strength standard solutions of NaCl were prepared. Prior to measurements, the polymeric solutions were filtered through 0.8 μm syringe filter whereas the solvent was filtered through a 0.45 μm Millipore filter. Separate Huggins and Kramer extrapolations were used to analyse results and the final values of $[\eta]$ were expressed in dl/g.

2.3. Steady shear flow measurements

Steady shear experiments on the sample solutions prepared at different concentrations were performed on a stress-controlled rheometer (Bohlin CS10) with a coaxial cylinders configuration (DG 40–50). The values of the shear stress–torque conversion constant, the shear rate-rotational speed conversion constant, the nominal gap and the inertia constant for this geometry are 3.437 Pa/Nm, 10.45 1/rad, 0 and 2.6×10^{-5} kgm^2, respectively. The shear stress as well as the shear rate vary slightly over the gap and the values given here for the constants refer to the average radius position. The measurements were carried out in the range of shear rates of 0.05–1000 s^{-1} and at $T = 30 \pm 0.1$ °C.

2.4. Dynamic measurements

Dynamic measurements were performed on the Bohlin CS10 using the same geometry as for the steady shear flow experiments discussed above. In frequency sweep measurements, the strain was fixed at 50% which was within the linear viscoelastic response established from strain sweep experiments.

The frequency of the applied strain wave was comprised in the range 0.001–20 rad/s.

These measurements gave us information on several viscoelastic parameters such as dynamic viscosity (η^*), storage modulus (G') and loss modulus (G'').

2.5. Fractionation of mannuronan

The mannuronan sample was fractionated by means of solvent/non-solvent fractionation according to the following procedure: to 5 g of the starting MANNA suspended in 100 ml of isopropanol was added 100 ml of distilled water. The mixture was stirred overnight in order to allow for the partial solubilization of the polymer. The suspension was centrifuged for one hour (5000 rpm), the resultant supernatant fluid was recovered and the organic phase removed on a rotatory evaporator. The solution was dialyzed (Wishing Tubes, cut off ≈ 12,000) and then freeze dried. The undissolved pellet was recovered and the procedure outlined above was iterated five more times, each time using a different volume ratio of water and isopropanol. In this way six fractions were obtained and then subjected to gel permeation chromatography analysis.

2.6. Gel permeation chromatography (GPC) coupled to triple detection array (TDA) for absolute molecular characterization

The size exclusion chromatography system consisted of two ViscoGel GMPWxl mixed bed columns. An online degasser was used to remove gas from the eluent. The mobile phase used was an aqueous solution of 0.1 M NaCl in distilled de-ionized water run at a flow rate of 0.8 ml/min. A volume of 100 μl of the MANNA solution was injected into the size exclusion system after filtering through a 0.45 μm Nylon filter (Millipore). The concentration of the sample was 0.6 mg/ml. A Viscotek Model 301 Triple Detector Array (TDA) which combines a concentration detector, viscometer and low angle light scattering detectors was used. It allows the determination of the absolute molecular weight, molecular structure, molecular size (to < 1 nm), branching and aggregation state. The detector alignment and instrument sensitivity parameters were previously determined by using a narrow and a broad polydispersed pullulan and dextran standards, respectively. The detector (RI, VISC, LS) temperature was set at 35 °C.

3. Results and discussion

3.1. Studies in the range of dilute solution

The results of the measurements carried out on dilute aqueous solutions of MANNA, MANNAEp4, MANNAEp1t5h and MANNAEp1t24h at different ionic strengths (I, M NaCl) and at 30 °C are displayed in Fig. 1. The relative viscosity values are reasonably confined within the range 1.2–2.0 for all tested concentrations. Combined application of both Huggins and Kramer extrapolations leads to an estimation of intrinsic viscosity $[\eta]$.

The dependence of K_H on I (Fig. 1, inserts) does not invoke changes in the macromolecular conformation due to the effect of I. Thus, no conformational transitions from a disordered state to an ordered one occurs by changing I.

By extrapolation of the reduced viscosity data to zero polymer concentrations (Fig. 1) the values of intrinsic viscosity, $[\eta]$, have been obtained. The intrinsic viscosity decreases regularly with increasing I (Fig. 2). At high ionic strengths the extent of intramolecular electrostatic repulsion decreases due to the effect of positive counter-ions screening on the negative-charged chain segments. Such behaviour is typical of dilute solutions of disordered

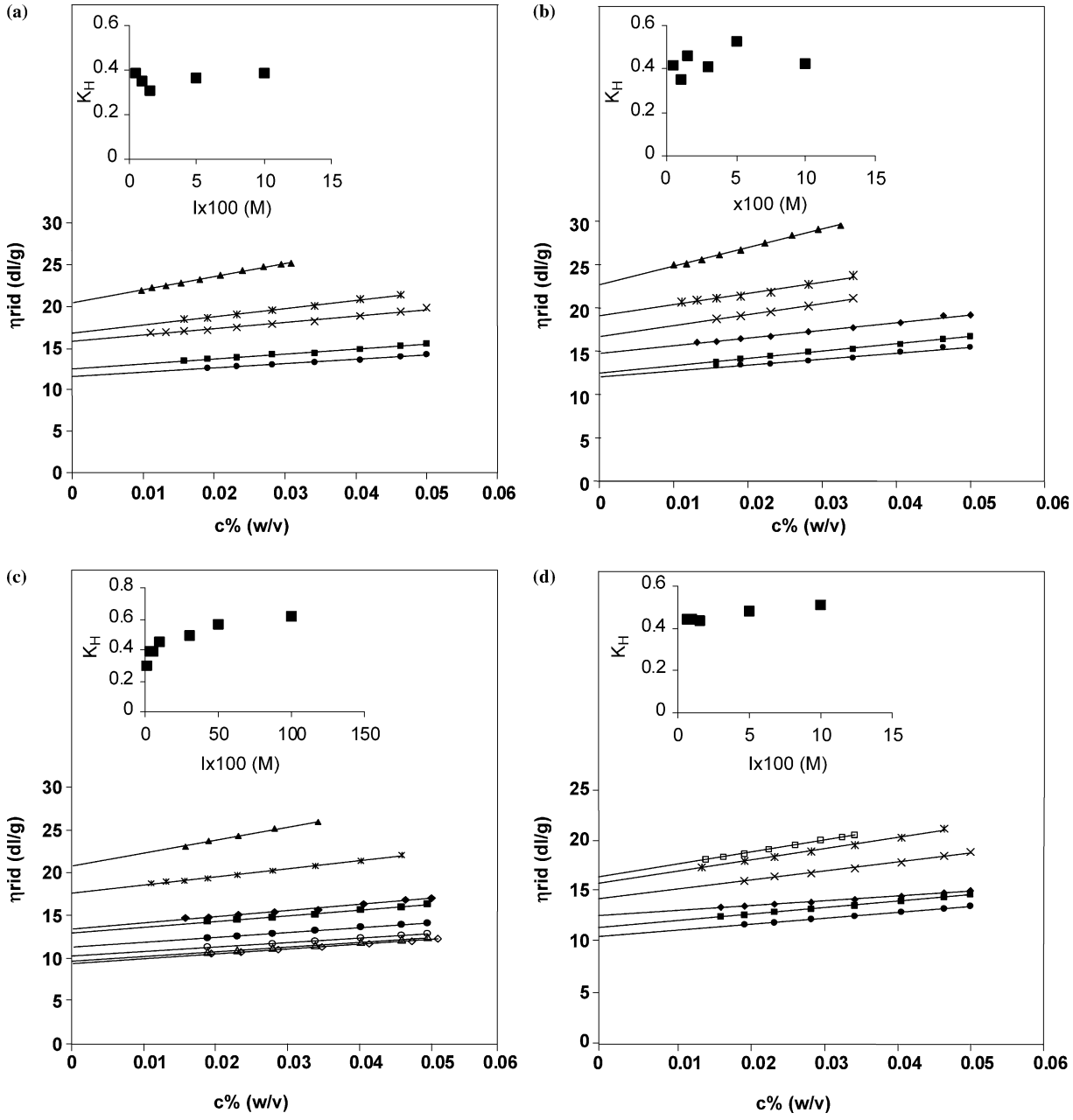

Fig. 1. Huggins extrapolations to intrinsic viscosity for (a) MANNA, (b) MANNAEp4, (c) MANNAEp1t5h and (d) MANNAEp1t24h at 30 °C. Ionic strengths (M, NaCl): (◇) 1, (△) 0.5, (○) 0.3, (●) 0.1, (■) 0.05, (♦) 0.03, (×) 0.015, (✱) 0.01, (□) 0.007, (▲) 0.005. Inserts: dependence of Huggins constant, K_H, on the ionic strength.

polyelectrolytes (Harding, 1997; Morris & Ross-Murphy 1987). This strengthens the hypothesis of polyelectrolytes in a disordered state.

The Smidsrød-Haug stiffness parameter B is a simple index for chain flexibility of polyelectrolytes. In the latest years new models have been proposed to evaluate the characteristics of stiffness/flexibility of charged biopolymers (Tobitani & Ross-Murphy, 1997). Nevertheless, the main advantage of the Smidsrød-Haug's B-value model is that the experimental procedure for the evaluation of this stiffness parameter is very simple: B-values can be determined only from $[\eta]$ data and no other parameter such as molecular weight is necessary.

As illustrated in Fig. 2, plots of intrinsic viscosity as a function of $I^{-1/2}$ for the polyelectrolytes here examined give a straight line whose slope (S) provides (once known the value of intrinsic viscosity at 0.1 M NaCl, $[\eta]_{0.1}$, taken as reference parameter) a valuation of B:

$$B = S/([\eta]_{0.1})^{1.3}$$

The B values calculated for the four samples examined in this study are listed in Table 2 which also reports the B

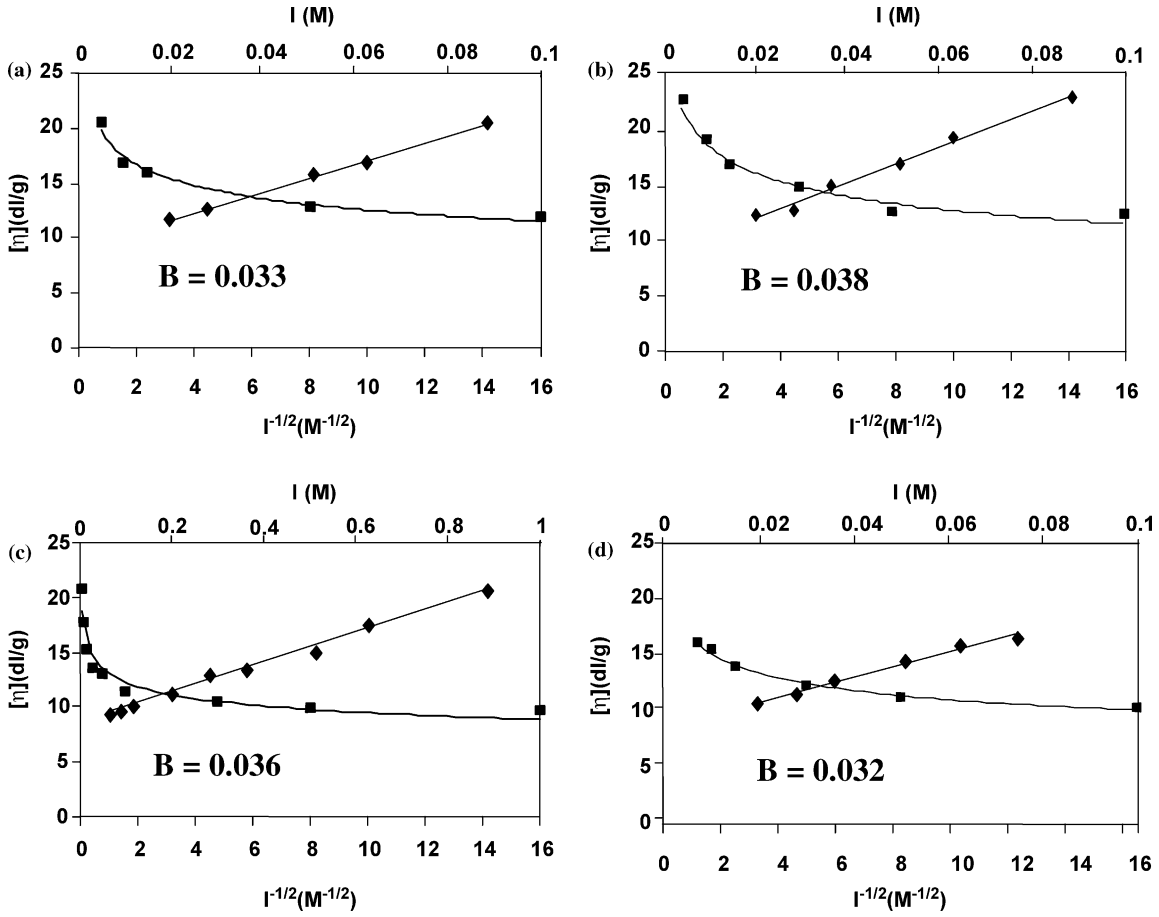

Fig. 2. Intrinsic viscosity, [η], of (a) MANNA, (b) MANNAEp4, (c) MANNAEp1t5h and (d) MANNAEp1t24h vs. ionic strength, I, and $I^{-1/2}$. ■: [η] vs. I, ♦: [η] vs. $I^{-1/2}$. From the slope (S) of the linear plot it is possible to obtain an estimate of the stiffness parameter B according to $B = S/([\eta]_{0.1})^{1.3}$, where $[\eta]_{0.1}$ is the intrinsic viscosity at $I = 0.1$ M NaCl.

values of several other polyelectrolytes arranged according to an increasing stiffness order. Locations of the B values of MANNA and of the epimerised mannuronans in such a list indicate that the polyelectrolytes at issue are of the semiflexible type.

Starting from MANNA ($B = 0.033$), it seems that epimerization by AlgE4, and hence the introduction of high fractions of MG diads, leads to an increase in the flexibility of the polymer chains ($B = 0.038$). This is in agreement with results previously reported by other studies (Stokke, Smidsrød, & Brant, 1993).

Epimerization by AlgE1 leads to a non-monotonous variation of the flexibility as a function of the enzyme's action time on the mannuronan substrate. The double epimerasic activity of AlgE1 unfolds in an initial increase in alternate sequences (leading to a concomitant increase in flexibility as it is shown by the case of MANNAEp1t5h, $B = 0.036$, Table 2). Longer epimerization times give rise to some decrease in the flexibility of the polymer chains as in the case of MANNAEp1t24h ($B = 0.032$). In the latter case the estimated B value is very similar to that obtained for a natural alginate rich in G-blocks (Table 2). This result corroborates both experimental findings and theoretical predictions.

3.2. Concentrated solution: steady shear measurements

Many polymers exist in solution as conformationally disordered random coils, whose shape fluctuates continually under Brownian motion. In dilute regime individual polymer

Table 2
The Smidsrød-Haug stiffness parameter B for some polyelectrolytes

Polyelectrolyte	B	Polyelectrolyte	B
Polyacrylate	0.230	MANNAEp4	0.038
Gellan in Me_4NCl (45 °C)	0.085	MANNAEp1t5h	0.036
CMC (DS = 1)	0.065	MANNA	0.033
Hyaluronic acid	0.065	MANNAEp1t24h	0.032
Oxidized (TEMPO) Konjac	0.055[a]	Alginate (high G content)	0.031
Oxidized (TEMPO) Detarium	0.050[a]	R. Trifolii EPS in NaCl	0.030
Oxidized (TEMPO) Salep	0.046[a]	DNA	0.006
Alginate (high M content)	0.040	Xanthan	0.005

The values of B for the pseudoalginates studied in this paper are also reported.

[a] Unpublished results from our laboratory, Department of Chemistry—University of Rome.

molecules are present as isolated coils, while in concentrated solutions the total hydrodynamic volume of the individual chains exceeds the volume of the solution. The concentration at which the transition from dilute to concentrated solution occurs depends on the hydrodynamic volume of the polymer molecules which in turn depends on the average molecular weight, the intrinsic characteristics of stiffness of the polymers and polymer–solvent interactions.

For random coil polymer solutions, intrinsic viscosity varies with coil dimensions according to Flory–Fox relationship (Morris, Cutler, Ross-Murphy, Rees, & Price, 1981). For any given polymer–solvent system the molecular weight of the dissolved macromolecules plays a crucial role in influencing the rheological properties. In order to correlate the rheological behaviour of the polymeric solutions with the intrinsic macromolecular properties of the polymers here investigated it is necessary that their molecular weights are comparable. The molecular weights of the four samples studied here are found to be quite similar (Table 1), thus allowing us to attribute differences in rheological behaviour solely to differences in molecular structure.

Strain sweep experiments were carried out in order to determine the linear viscoelastic range of the studied polysaccharide solutions. The response of semi-dilute pseudoalginates solutions to steady shear rate experiments were studied over a wide range of concentrations (0.1–1.2% w/v). Fig. 3 shows the shear rate viscosity vs. shear rate in a range 10^{-2} to 10^{2} s^{-1} on a double logarithmic scale. No shear rate viscosity dependence was shown in the range of γ used for concentrations lower than 0.3% w/v. However, for solutions of concentrations above this level, obvious shear thinning was found, and as expected, this shear thinning became more pronounced as the concentration is increased.

In the literature some rheological models are reported to describe the viscosity of many polymeric fluids showing Newtonian behaviour at low $\dot{\gamma}$ and a power-law behaviour at high shear-rates (Wang, Ellis, Ross-Murphy, & Burchard, 1997).

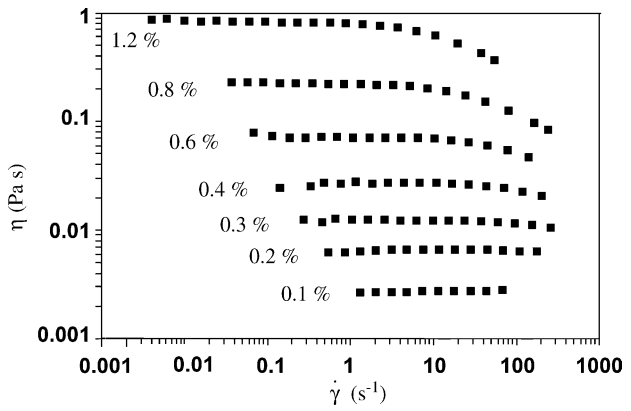

Fig. 3. Shear rate ($\dot{\gamma}$) dependence of viscosity (η) for different concentrations (%, w/v) as shown in figure of MANNA solutions at ionic strength equal to 0.1 M (NaCl) and $T = 30$ °C.

From plots of Fig. 3 it is possible for the different polymer concentrations, to extrapolate the zero shear rate viscosities. From these data, the concentration dependence of the zero-shear rate viscosity (η_{sp}) can be presented conveniently as a double logarithmic plot of η_{sp} against the coil overlap parameter $c[\eta]$ where c is the polymer concentration (Fig. 4). Two different behaviours characterised by different slopes were detected. Typically, these two domains define two regimes: the dilute one, obtained at low values of the coil overlap parameter, and the semi-dilute regime at higher coil overlap values. In Fig. 4 the experimental points (empty symbols) at lower concentrations were obtained using the glass capillary viscometer while the rest of the data were obtained with the Bohlin rheometer. The first regime with a slope of 1–1.2 corresponds to dilute solution, and the second one with a slope of 3–3.5 represents semi-dilute solutions.

As it can be observed, the variation in rheological behaviour is more sharp in the case of MANNA and MANNAEp4. The slope values reported for concentrated solutions are typical of most linear polymers forming topologically entangled networks.

From the intersection between the extrapolations of the two straight lines it is possible to calculate the coil overlap parameter $c^*[\eta]$ and, thus, the critical concentration c^*.

The coil-overlapping values are 3.1, 3.2, 2.2, 2.0 and the relative critical concentrations are reported in Fig. 4.

Morris et al. (1981) observed that for several polysaccharide solutions the concentration dependence of zero-shear viscosity on the parameter $c[\eta]$ follows the same general behaviour. Values of $c^*[\eta]$ lying in the range 3–6 are typical of most polysaccharides.

Somewhat lower values have been found for relatively stiff and extended chains (e.g. hyaluronate under conditions of low pH and high ionic strength) (Morris, Rees, & Welsh, 1980). The coil overlap value found for MANNAEp1t24h confirms the hypothesis of relatively stiff chain if compared to the other polyuronans we have examined. It is worth noting that the increasing amount of G residues, and hence the average length of G-blocks, influences the coil overlapping parameter to a very little extent passing from MANNAEp1t5h to MANNAEp1t24h.

It must be stressed that although the molecular weight of MANNAEp4 is higher than those relative to MANNAEp1t5h and MANNAEp1t24h, it turns out that the critical concentration value of the MG-structured polymer is higher than the c^*s of the samples epimerized by AlgE1. Such experimental evidence supports the hypothesis of a higher flexibility of MANNAEp4 which leads to an effective folding of the chains and consequently to lower hydrodynamic volumes. These results are in agreement with the conformational energy results found for the four type of dimers occurring in alginates (Stokke, Smidsrød, & Brant, 1993). In particular, the G–G and G–M potential energy maps suggest a lower degree of

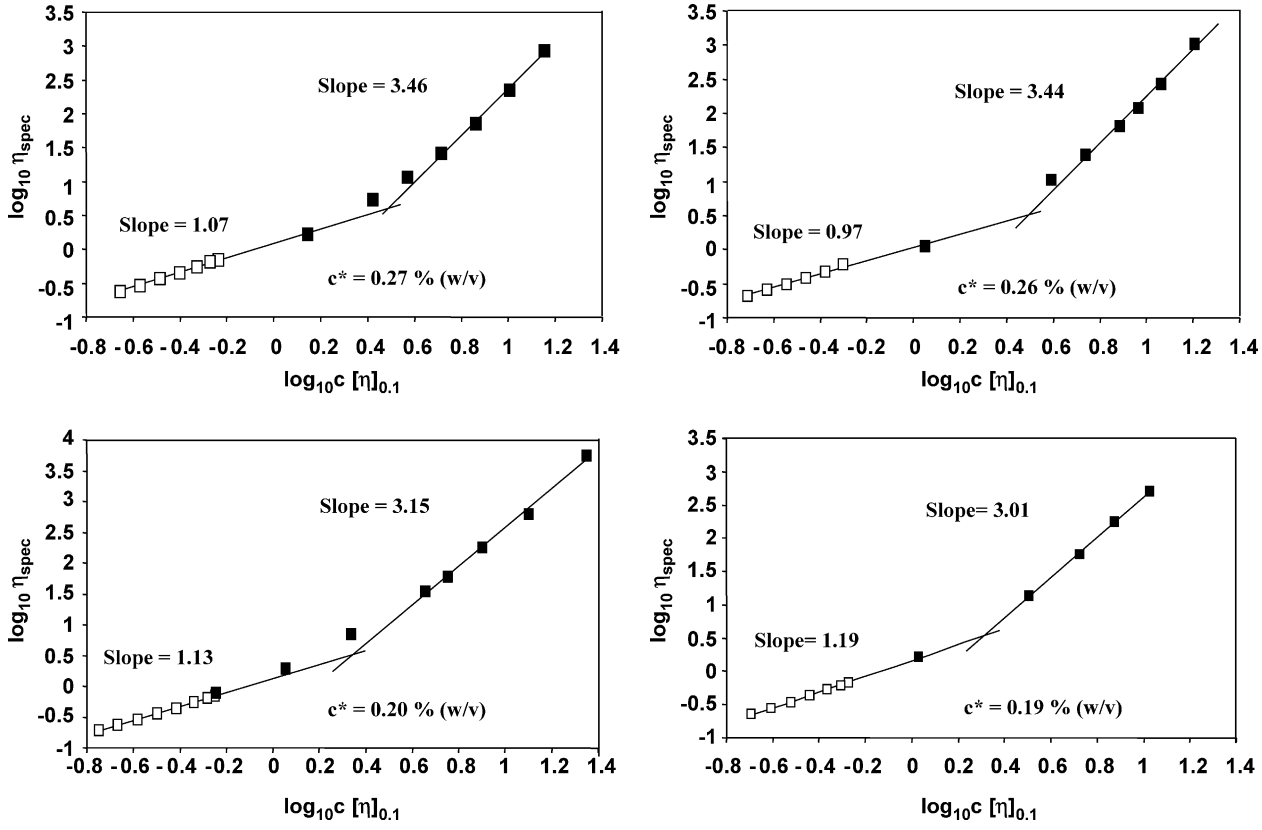

Fig. 4. Variation in the 'zero shear' viscosity of (a) MANNA, (b) MANNAEp4, (c) MANNAEp1t5h and (d) MANNAEp1t24h solutions at 30 °C and $I=0.1$ M NaCl. Experimental points (■) were obtained by using the Bohlin rheometer. Range of concentrations (%, w/v): (a) 1.23–0.12, (b) 1.32–0.09, (c) 1.98–0.05 and (d) 1–0.1. Other experimental points (□) were obtained by using the glass capillary viscometer. Range of concentrations (%, w/v): (a) 0.05–0.019, (b) 0.041–0.016, (c) 0.05–0.016 and (d) 0.05–0.019. The critical concentration value is provided by the intersection of the straight lines.

conformational freedom around the glycosidic linkages than in M–G and M–M disaccharides.

Our studies in concentrated solution demonstrate that the critical concentration of MANNA is actually similar to the c^* obtained for MANNAEp4, thus strengthening the hypothesis of similar folding, and hence similar potential energy restraints for these pseudo-alginates.

On the basis of the B parameter values, it would be a logical conclusion to predict a c^* for MANNA lower than the one found for MANNAEp4. On the contrary, both our experimental results and computer modelling studies on MM and MG disaccharides, which resulted in almost super imposable potential maps, indicate that the two types of sequences enjoy the same set of conformational states. Such evidence points out the main limit of the B-parameter method: it estimates the ability of polyelectrolyte coils to expand or contract in response to change in ionic strength but it does not delineate the characteristics of flexibility of the starting system.

3.3. Concentrated solution: oscillatory shear measurements

The dependence of G^* on strain for MANNA and epimerized mannuronans solutions was determined by strain-sweep experiments. An example of the plots obtained is displayed in Fig. 5. It can be seen that G^* is independent of the strain at the two concentrations examined, up to $\gamma \sim 100\%$. Therefore, all the following frequency sweep experiments were carried out at $\gamma = 50\%$.

Tests carried out under oscillatory conditions have provided the ω dependence of the dynamic viscosity η^*.

The Cox–Merz rule states that the magnitudes of the complex viscosity ($\eta^*(\omega)$) and the steady shear viscosity ($\eta(\dot{\gamma})$) must be equal at equal values of frequency and shear rate. The experimental verification of the convergence

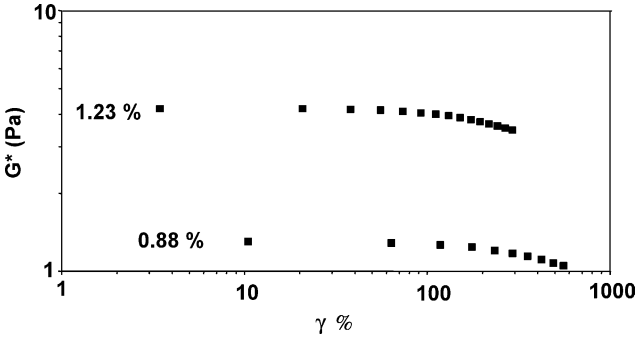

Fig. 5. Dependence of G^* on strain γ (%) for 0.88 and 1.23% (w/v) MANNA solutions (0.1 M NaCl, $T=30$ °C, $\omega=1$ Hz).

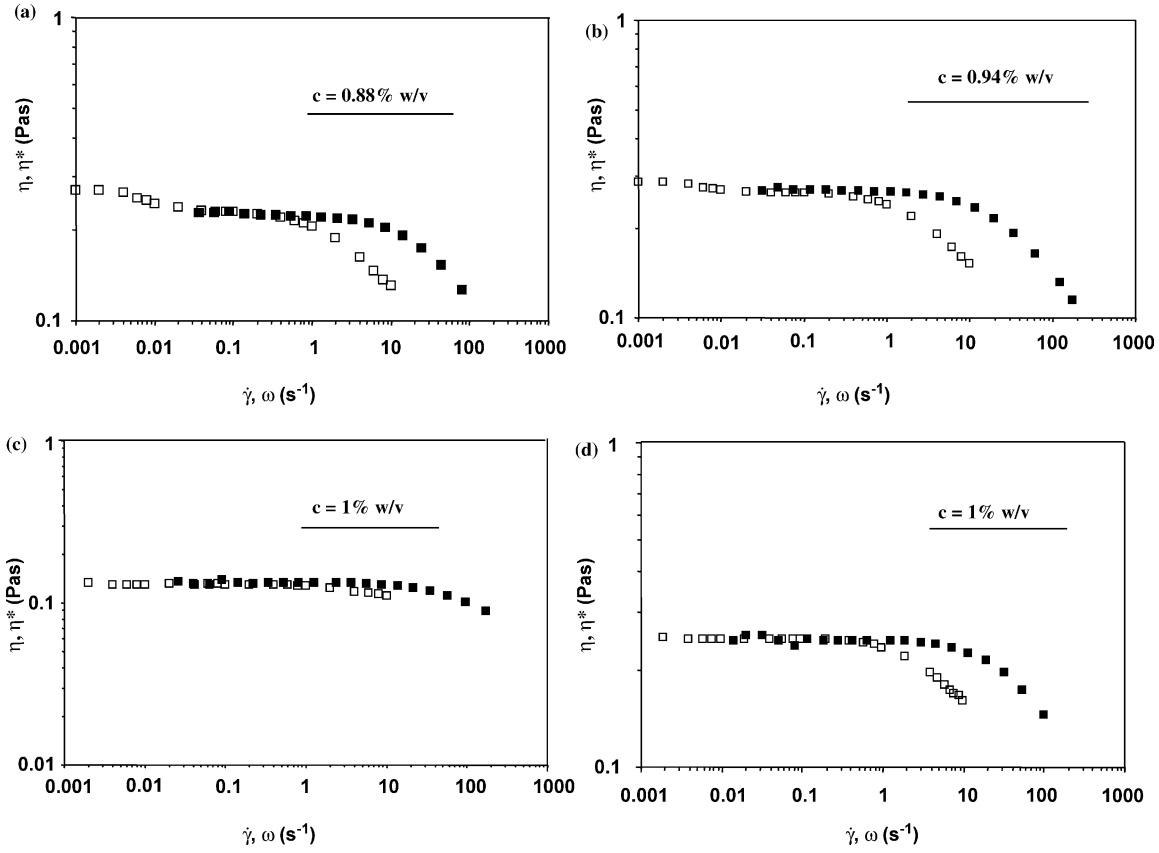

Fig. 6. Steady shear viscosity η (■) and complex dynamic viscosity (□) plotted against shear rate ($\dot{\gamma}$) and corresponding frequency (ω) for (a) MANNA, (b) MANNAEp4, (c) MANNAEp1t5h and (d) MANNAEp1t24h. Concentrations (%, w/v): (a) 0.88, (b) 0.94, (c) 1 and (d) 1. Dynamic oscillatory tests were performed setting $\gamma = 50\%$. $T = 30$ °C.

condition ($\eta^*(\omega) = \eta(\dot{\gamma})$) is further evidence of the solution state of a polymeric system and for discrimination between polymer solution and structured systems. In the present case, the empirical Cox–Merz rule (Cox & Merz, 1958) is only partially verified (Fig. 6). Both $\eta(\dot{\gamma})$ and $\eta^*(\omega)$ approach the same limit value η_0 as $\dot{\gamma}$ and ω tend towards zero. In the Newtonian region the chains essentially behaves like disordered coils whose relaxation times are largely shorter than the time-scale of the external imposed perturbation, thus leading to a close adherence to the Cox–Merz rule.

At higher $\dot{\gamma}$ and ω the divergence between $\eta(\dot{\gamma})$ and $\eta^*(\omega)$ curves (with the partial exception of MANNAEp1t5h) becomes more and more pronounced. Such behaviour points to a more effective disruption effect on a network structure under either oscillatory or steady shear and it has been observed elsewhere (Lapasin, Pricl, & Tracanelli, 1991; Richardson & Ross-Murphy, 1987). For instance, it has been noticed that the Cox–Merz rule essentially fails in the case of systems characterized by the occurrence of specific intermolecular interactions as, for instance, concentrated xanthan solutions. Their rheological response approaches the typical weak gel viscoelastic behaviour (Oviatt & Brant, 1993).

In our case, the non-super imposable traces of $\eta(\dot{\gamma})$ and $\eta^*(\omega)$ at high $\dot{\gamma}$ and ω might be due to a sort of *perturbation* of the statistical coil state caused by the imposed deformation, leading to a disruption of the non-specific intermolecular interactions in favour of more specific inter-chain couplings between pseudoalginate chains.

This behaviour may be explained by making reference to the residue composition of MANNA and the pseudoalginates here examined (Table 1) and assuming that the regularity in the residue sequence composition is a pre-requisite for efficient intermolecular chain interaction. Among the polysaccharides shown in Table 1, MANNA is a homopolymer and as a consequence enjoys a very regular primary structure. MANNAEp4 has a well defined alternate MG sequence: the proportion of M and G residues is very close to each other and the fractions of MM and GG diads are negligible (Table 1). On the contrary, pseudo-alginates obtained by AlgE1 present a more heterogeneous primary structure. However, MANNAEp1t24h is constituted by very long G-blocks while the fraction of MG diads is rather low. It may be envisaged that these G-block stretches may give rise to efficient intermolecular interactions as evidenced by the deviation from the Cox–Merz rule at high shear rates and frequencies. MANNAEp1t5h residue sequence is far

less ordered if compared with MANNA and MANNAEp4 and also with respect to MANNAEp1t24h: the extension of G blocks is markedly lower than that of MANNAEp1t24h and it can be inferred from data of Table 1 that the contribution of M and MG blocks fraction are more pronounced. A more disordered primary structure may be reflected in a larger set of accessible conformations of MANNAEp1t5h which in turn may cause at high rates of deformation a less extended occurrence of specific chain–chain interactions, thus not essentially perturbing the macromolecule's initial shape and dimensions (in the range of shear rates and frequencies instrumentally allowed).

3.4. Characteristics of flexibility of mannuronan: a TDA–gel permeation chromatography (GPC) study

In Table 3 the number average molecular weight (M_n), the weight average molecular weight (M_w) and the polydispersity index (Pd = M_w/M_n) determined by trial detector GPC for the fractionated MANNA sample selected are shown.

Fractionation based upon solvent/non-solvent method proved itself to be quite efficient in reducing the polydispersity of the starting polysaccharide (Pd = 6.8).

3.4.1. Mark–Houwink–Sakurada relationship

A sample solution of the fractioned mannuronan was run through the GPC column and at the output the triple detector permits an actual correlation between the molecular weight of the eluting sample and its intrinsic viscosity. In the range of molecular weights reported, the continuous trace of $[\eta]$ vs. M_w (double logarithmic scale) results in a straight line, whose slope and intercept provide an estimation of the Mark–Houwink–Sakurada parameters, α and K, respectively ($\alpha = 0.73$ and $K = 1.3 \times 10^{-3}$ dl/g) (Fig. 7).

Random coil flexible chains usually have an α in the range 0.5–0.8 (Lapasin & Pricl, 1995). Polyelectrolytes show values of α close to 0.8 because of the effect of the charged residues, leading to a large increase in α when the ionic strength decreases (Smidsrød, 1970).

The value of α estimated for the MANNA sample is quite similar to the ones reported in the literature for other polyelectrolytes such as hyaluronic acid (Cleland & Wang, 1970), pectins (Axelos, Thibault, & Lefebvre, 1989) and carboxymethylcellulose (Brown & Henly, 1964).

Table 3
Quantitative results of the analysis performed on the MANNA sample obtained by means of solvent/non-solvent fractionation from a starting mannuronan with Pd = 6.8

M_w (D)	M_n (D)	Pd = M_w/M_n	$[\eta]$ (dl/g)
1.8×10^4	1.0×10^4	1.71	8.73

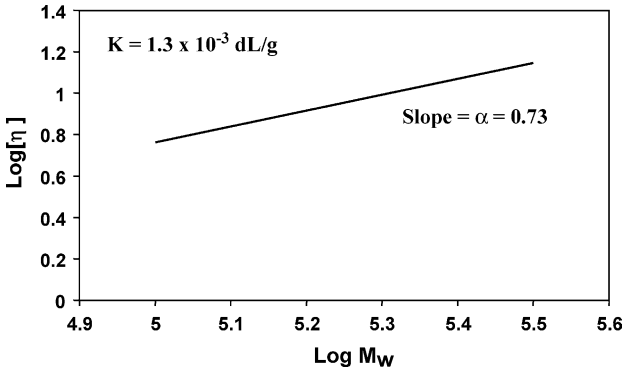

Fig. 7. Mark–Houwink–Sakurada plot ($\log[\eta]$ vs. $\log M_w$) for the MANNA sample with Pd = 1.71. The parameters α and K are 0.73 and 1.3×10^{-3} dl/g, respectively.

3.4.2. The Stockmayer–Fixman equation and the characteristic ratio C_∞

The Stockmayer–Fixman equation permits through an extrapolation to determine the molecular dimension of an unperturbed coil from the perturbed one, on condition that the intrinsic viscosity of the sample is known in a large range of molecular weights.

Such equation is based upon the general principle that the segment–segment long distance interactions will decrease with a decrease of the chain length, thus leading the dimensions of the perturbed and unperturbed coils to convergence when M_w tends to 0.

The Stockmayer–Fixman equation is defined as

$$[\eta]/M^{1/2} = K_\theta(1 + C_1 M^{1/2})$$

and it is equivalent to the Mark–Houwink–Sakurada for an unperturbed random coil, with an additional term proportional to M, which takes into account the long distance effects. The intercept K_θ, which corresponds to a polymeric chain in the θ-state, i.e. where there is no excluded volume, is proportional to the characteristic ratio C_∞ according to

$$K_\theta = \Phi I^3 (C_\infty/m_0)^{3/2}$$

where Φ is the Flory's universal constant (2.6×10^{26} kg^{-1}) while l and m_0 stand for the length and the weight of the monomeric unit, respectively, and C_∞ is the characteristic ratio defined as

$$C_\infty = \langle R^2 \rangle / N l^2$$

$\langle R^2 \rangle$ is the mean squared end-to-end distance for the polymer chain and N the number of residues along the chain. The product Nl^2 is the mean squared end-to-end distance for an ideal chain, with freely jointed segments and no conformational restrictions. Therefore, the characteristic ratio C_∞ is an estimate of the stiffness of the real chain and hence of the degree of directional correlation between neighboring residues.

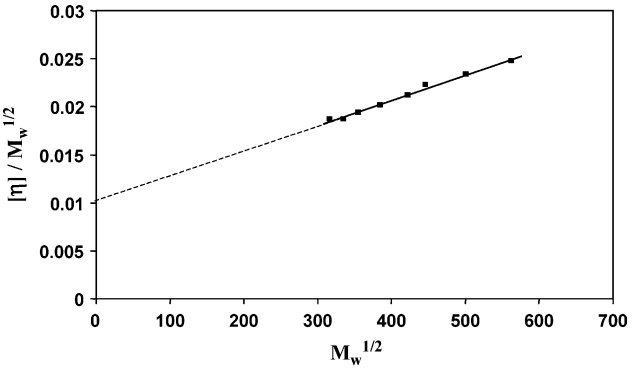

Fig. 8. Stockmayer–Fixman plot for the MANNA sample with Pd = 1.71. The calculated characteristic ratio was $C_\infty = 16.8$.

In Fig. 8 the Stockmayer–Fixman plot for the MANNA sample is shown. The intercept ($K_\theta = 0.010$ dl/g) gives a $C_\infty = 16.8$ assuming $l = 0.54$ and $m_0 = 198$.

This value is somewhat higher than the one obtained for hyaluronic acid ($C_\infty = 13.1$, unpublished data from our laboratory) a polyelectrolyte with a lower charge density than MANNA. On the other end, in the case of guar galactomannan and hydroxypropyl guar (uncharged polymers) values of C_∞ of 14.7 ± 1.2 (Picout, Ross-Murphy, Errington, & Harding, 2002) and 13.02 (Cheng, Brown, & Prud'homme, 2002), respectively, have been reported. For pullulan (Buliga & Brant, 1987) and amylose (Brant & Min, 1969) values of $C_\infty = 5$ and 10, respectively, are reported. The higher value of the characteristic ratio of MANNA compared to that of hyaluronic acid seems to be due both to a higher intrinsic chain rigidity but also can be explained by the effect of electrostatic repulsion among charged chain segments which causes an expansion of the coil dimensions and yields a stiffer macromolecular conformation than galactomannans.

The chain persistence length, L_p, can be obtained by the relationship $C_\infty = 2L_p/l$ valid in the case of flexible worm-like coils. In our case, $L_p = 4.5$ nm. Such value lies in the range of chain persistence lengths (3–5 nm) typical of some β-(1–4)-linked *O*-glycosidic polymeric backbones reported in literature (Picout & Ross-Murphy, 2002; Picout, Ross-Murphy, Jumel, & Harding, 2002; Muroga, Yamada, Noda, & Nagasawa, 1987; Valtasaari, 1971).

We may, therefore, conclude that, in spite of its charged nature, the conformational behaviour of MANNA is quite similar to that of several non-charged polysaccharides such as galactomannans and arabinoxylans.

It is interesting to compare the results obtained for MANNA to some past published works dealing with the chain flexibility of alginates. Conformational energy calculations on alginic acid yielded a $C_\infty = 16$ and $L_p = 4.2$ nm for the alternating MG copolymer and considerably higher values of the characteristic ratio for poly-M and poly-G sequences (Whittington, 1971). On the basis of our work, it seems that chain stiffness appear to be less influenced by differences in the content of M and G residues than previously estimated. This is in agreement with conformational energy results (Stokke, Smidsrød, & Brant, 1993) which foresee for alginates containing a large fraction of poly(MG), a less extended conformation than those rich in M, while those rich in G are more extended. Anyway, differences are not particularly relevant and this is in agreement with our findings.

The Kuhn length can be calculated from C_∞ using the expression

$$A_m = (C_\infty + 1)l$$

It turns out that $A_m = 9.6$ nm, somewhat lower than the Kuhn dimensions calculated for natural alginates on the basis of light scattering and viscosity measurements (Smidsrød, Glover, & Whittington, 1973).

4. Conclusions

Both capillary viscometry and rheology techniques have enabled us to characterize solutions of mannuronan and mannuronan epimerized by AlgE1 at different times and AlgE4. This study focused on the properties of flexibility of the pseudoalginates chain backbone, a feature which influences at higher concentrations the transition from dilute to semi-dilute regime.

The presence of significant intermolecular interactions occurring in concentrated solutions tends to causes a significant deviation from Cox–Merz rule at high rates of deformation.

The exponential parameter α of the Mark–Houwink equation and the Stockmayer–Fixman characteristic ratio calculated for MANNA are typical of expanded polyelectrolytes, whose segments tends to depart from each other because of electrostatic repulsive interactions.

The characteristic ratio C_∞, the persistence length L_p and the Kuhn length A_m have been calculated providing together with other works previously published information about the flexibility of the polymannuronic backbone.

Acknowledgements

This work has been carried out with the financial support of the European Union (contract QLK3-CT-1999-00034).

We are grateful to Nicola Marheineke, application chemist at Viscotek–Europe, Kingsclere Rd., Chapel House, Basingstoke for the GPC measurements on MANNA samples.

References

Axelos, M. A. V., Thibault, J. F., & Lefebvre, J. (1989). Structure of citrus pectins and viscosimetric study of their solution properties. *International Journal of Biological Macromolecules*, *11*(3), 186–191.

Braccini, I., & Pérez, S. (2001). Molecular basis of Ca^{2+}-induced gelation in alginates and pectins: the Egg–Box model revisited. *Biomacromolecules*, *2*(4), 1089–1096.

Brant, D. A., & Min, B. K. (1969). The unperturbed dimensions of sodium carboxymethylamylose. *Macromolecules*, *2*, 1–8.

Brown, W., & Henley, D. (1964). Cellulose derivatives. IV. The configuration of the poly-electrolyte sodium carboxymethyl cellulose in aqueous sodium chloride solutions. *Makromolekulare Chemie*, *79*, 68–88.

Buliga, G. S., & Brant, D. A. (1987). Temperature and molecular weight dependence of the unperturbed dimensions of aqueous pullulan. *International Journal of Biological Macromolecules*, *9*, 71–77.

Cheng, Y., Brown, K. M., & Prud'homme, R. K. (2002). Characterization and intermolecular interactions of hydroxypropyl guar solutions. *Biomacromolecules*, *3*, 456–461.

Cleland, R. L., & Wang, J. L. (1970). Ionic polysaccharides. 3. Dilute solution properties of hyaluronic acid fractions. *Biopolymers*, *9*(7), 799–810.

Cox, W. P., & Merz, E. H. (1958). Correlation of dynamic steady flow viscosities. *Journal of Polymer Science*, *28*(118), 619–622.

Donati, L., Coslovi, A., Gamini, A., Skjåk-Bræk, G., Veter, A., Campa, C., & Paoletti, P. (2004). Galactose substituted Alginate 2: conformational aspects. *Biomacromolecules*, *5*, 5186–11196.

Draget, K. I., Skjåk-Bræk, G., & Smidsrød, O. (1997). Alginate based new materials. *International Journal of Biological Macromolecules*, *21*, 47–55.

Ertesvåg, H., Hoidal, H. K., Hals, I. K., Rian, A., Doseth, B., & Valla, S. (1995). A family of modular type mannuronan C-5 epimerases genes controls alginate structure in *Azotobacter vinelandii*. *Molecular Microbiology*, *16*(4), 719–731.

Ertesvåg, H., Høydal, H., Skjåk-Bræk, G., & Valla, S. (1998). The *Azotobacter vinelandii* mannuronan C-5 epimerase AlgE1 consists of two separate catalytic domains. *Journal of Biological Chemistry*, *273*, 30927–30938.

Ertesvåg, H., & Skjåk-Bræk, G. (1999). Modification of alginate using mannuronan C-5-epimerases. *Methods in Biotechnology*, *10*, 71–78.

Ertesvåg, H., & Valla, S. (1998). Biosynthesis and applications of alginates. *Polymer Degradation and Stability*, *59*, 85–91.

Gimmestad, M., Sletta, H., Ertesvåg, H., Bakkevig, K., Sumita, J., Skjåk-Bræk, G., et al. (2003). The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5 epimerase activity, is needed for alginate polymer formation. *Journal of Bacteriology*, *185*, 3515–3523.

Grasdalen, H. (1983). High-field ^{1}H-NMR spectroscopy of alginate: sequential study and linkage conformations. *Carbohydrate Research*, *118*, 255–260.

Harding, S. E. (1997). The intrinsic viscosity of biological macromolecules. Progress in measurement, interpretation and application to structure in dilute solution. *Progress in Biophyics and Molecular Biology*, *68*(2/3), 207–262.

Hartmann, M., Holm, O. B., Johansen, G. A. B., Skjåk-Bræk, G., & Stokke, B. T. (2002). Mode of action of recombinant *Azotobacter Vinelandii* mannuronan C-5 epimerases from AlgE2 and AlgE4. *Biopolymers*, *63*, 77–88.

Høydal, H., Ertesvåg, H., Stokke, B. T., Skjåk-Bræk, G., & Valla, S. (1999). Biochemical properties and mechanism of action of the recombinant *Azotobacter vinelandii* mannuronan C-5 epimerase. *Journal of Biological Chemistry*, *274*, 12316–12322.

Lapasin, R., & Pricl, S. (1995). *Rheology of industrial polysaccharides. Theory and applications*. Academic & Professional.

Lapasin, R., Pricl, S., & Tracanelli, P. (1991). Rheology of hydroxyethyl guar gum derivatives. *Carbohydrate Polymers*, *14*(4), 411–427.

Morris, E. R., Cutler, A. N., Ross-Murphy, S. B., Rees, D. A., & Price, J. (1981). Concentration and shear rate dependence of viscosity in random coil polysaccharide solutions. *Carbohydrate Polymers*, *1*(1), 5–21.

Morris, E. R., Rees, D. A., & Welsh, E. J. (1980). Conformation and dynamic interactions in hyaluronate solutions. *Journal of Molecular Biology*, *138*(2), 383–400.

Morris, E. R., & Ross-Murphy, S. B. (1987). Chain flexibility of polysaccharides and glycoproteins from viscosity measurements. *Techniques in Carbohydrate Metabolism*, *B310*, 1–46.

Muroga, Y., Yamada, Y., Noda, I., & Nagasawa, M. (1987). Local conformation of polysaccharides in solution investigated by small-angle X-ray scattering. *Macromolecules*, *20*, 3003–3007.

Oviatt, H. W., & Brant, D. A. (1993). Thermal treatment of semidilute aqueous xanthan solutions yields weak gels with properties resembling hyaluronic acid. *International Journal of Biological Macromolecules*, *15*, 3–10.

Picout, D. R., & Ross-Murphy, S. B. (2002). On the chain flexibility of arabinoxylans and other β-(1→4) polysaccharides. *Carbohydrate Research*, *337*, 1781–1784.

Picout, D. R., Ross-Murphy, S. B., Errington, N., & Harding, S. E. (2002a). Pressure cell assisted solution characterization of polysaccharides. 1. Guar Gum. *Biomacromolecules*, *2*(4), 1301–1309.

Picout, D. R., Ross-Murphy, S. B., Jumel, C., & Harding, S. E. (2002b). Pressure cell assisted solution characterization of polysaccharides. 2. Locust Bean Gum and Tara Gum. *Biomacromolecules*, *3*(4), 761–767.

Richardson, R. K., & Ross-Murphy, S. B. (1987). Non-linear viscoelasticity of polysaccharide solutions. 2: Xanthan polysaccharide solutions. *International Journal of Biological Macromolecules*, *9*, 257–264.

Skjåk-Bræk, G., & Espevik, T. (1996). Application of alginate gels in biotechnology and biomedicine. *Carbohydrates in Europe*, *14*, 19–25.

Smidsrød, O. (1970). Solution properties of alginate. *Carbohydrate Research*, *13*(3), 359–372.

Smidsrød, O., Glover, R. M., & Whittington, S. G. (1973). Relative extension of alginates having different chemical composition. *Carbohydrate Research*, *27*(1), 107–118.

Stokke, B. T., Smidsrød, O., & Brant, D. A. (1993). Predicted influence of monomer sequence distribution and acetylation on the extension of naturally occurring alginates. *Carbohydrate Polymers*, *22*, 57–66.

Stokke, B. T., Smidsrød, O., Bruheim, P., & Skjåk-Bræk, G. (1991). Distribution of uronate residues in alginate chains in relation to alginate gelling properties. *Macromolecules*, *24*, 4637–4645.

Tobitani, A., & Ross-Murphy, S. B. (1997). The intrinsic viscosity of polyelectrolytes revisited. *Polymer International*, *44*, 338–347.

Valtasaari, L. (1971). Configuration of cellulose dissolved in iron–sodium tartrate. *Makromolekulare Chemie*, *150*, 117–126.

Wang, Q., Ellis, P. R., Ross-Murphy, S. B., & Burchard, W. (1997). Solution characteristics of the xyloglucan extracted from *Detarium Senegalense Gmelin*. *Carbohydrate Polymers*, *33*, 115–124.

Whittington, S. G. (1971). Conformational energy calculations on alginic acid. II. Conformational statistics of the copolymers. *Biopolymers*, *10*(9), 1617–1623.