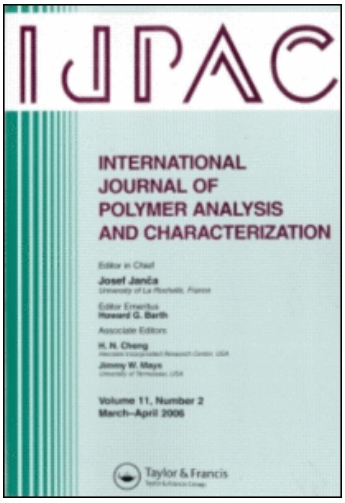

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Characterization of an Aqueous-Dissolved Anionic Polysaccharide Using Rheology and Size-Exclusion Chromatography with a Differential Refractive-Index and Low-Angle Laser-Light Scattering Detectors

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From the aqueous growth medium of Red Microalgae *Porphyridium sp.*, anionic polysaccharide was separated by centrifugation and dialysis. Xylose, glucose, galactose, mannose, rhamnose, arabinose, methyl-pentoses, dimethyl-hexoses, glucuronic acid and sulfated sugar-units were identified as constituting monomers. By means of size-exclusion chromatography with differential refractive index and low-angle laser-light scattering (DRI/LALLS) detectors, an average molecular weight of 8.4×10^6 g/mol was determined. For the investigated sample concentrations in the range of 0.08–0.1 mg/mL, SEC separation based on decreasing molecular weight was not observed; however, decreasing particle/fragment dimensions with increasing retention volume were eluted. Rheological investigations at 20°C showed that an aqueous 0.05 M NaCl solution of the dissolved polysaccharide was non-Newtonian with high elasticity. By comparing the polymer-coil packing density of the anionic polysaccharide with three reference polysaccharides, a particle/fragment-packing similar to dextran coils was found. With information about packing density and absolute molecular weight, universal calibration enabled an estimation of dimensions for the constituting components of the anionic polysaccharide in terms of sphere-equivalent radii. The SEC-eluted polymer-coils were found in the range between ≈2–140 nm with a maximum mass occurring at ≈90 nm.

KEY WORDS Anionic polysaccharide, rheology, size-exclusion chromatography, low-angle laser light scattering

INTRODUCTION

The most difficult and thus, the most important step in the characterization of polymers from biological sources, like microbes or plants, is their isolation and purification without generating artefacts, which is even more important when molecular characteristics are to be correlated with native biological functions [1–3]. The major problem in such investi-

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gations is the fact, that once an isolation procedure includes any kind of denaturation step (e.g., desalting or freeze-drying) initial native structures will be lost, more or less completely. This is because native structures are generated and controlled by complex dynamic equilibria within the solution, (changing) environmental conditions, specific particle geometries/dimensions, and by a range of other physical and chemical interactions. Therefore, even if there is good evidence that initially some basic polymer structure has been conserved by adequate isolation procedures, there is no way to predict the degree of restoration of these native structures after, for example, redissolving of a freeze-dried biopolymer [4–6].

An important group of such substances with a wide range of biological functions and an even wider range of structural and interactive properties are polysaccharides [7]. These renewable biopolymers, on the one hand, are of increasing interest as low-cost raw-materials for industrial purposes, but, on the other hand, processing is too expensive because of enormous fluctuations in the quality and properties of the supplied raw-materials. To help remove this uncertainity, comprehensive chemical, physical and biological characterization of polysaccharides and polysaccharide-containing systems are required.

One of the topics, which is of particular interest, is the capability of aqueous-dissolved polysaccharides to form polymer/polymer interactions which are responsible for association, aggregation and gelation. As a matter of fact, polymer/polymer interactions generate and control many characteristic dimensions within polymer/solvent systems, such as the formation of reversible/dynamic associates which lead to supramolecular time-dependent structures [8,9]. Gel-forming aqueous polysaccharide solutions show weak interactions, as in the case of particle solutions close to thermodynamic equilibrium, as well as strong interactions, as in the case of chaotic systems far from thermodynamic equilibrium with macroscopic coherence in geometry and time-scale. Within this range of characteristics nonreproducible supramolecular structures and pronounced sensitivity to even the slightest changes in environmental conditions, shift aqueous polysaccharide solutions from simple thermodynamic equilibrium towards complex dynamic equilibria. Although structures formed by such systems could be transformed and/or destroyed by excessive applied external forces (e.g., during isolation or purification), there remains the potential within the system to regenerate similar structures again. This basic capability can be correlated with specific molecular characteristics, such as the width of molecular weight distribution, packing density of polysaccharide coils, and particle and fragment dimensions, which can be studied by means of physicochemical techniques like chromatography, scattering methods, and rheology.

A denaturated/renaturated polysaccharide produced by Red Microalgae *Porphyridium sp.* was ingestigated *in vitro* and found to contain a fascinating mixture of different particle and fragment sizes. These anionic polysaccharides are extracted from from red algae and are used in food industry as thickeners and gelling agents. Their biological function is also of interest, because they cause significant resistance to mass transfer in aqueous medium to growing algae cells. The retardation effects of polysaccharide gels on the transfer of ions and nutrients and the dependence of mass transfer on the interaction between fixed cell-capsulating and dissolved polysaccharides is known, but no correlation between these functions which inhibit the growth rates of algae cells and physicochemical characteristics has been established up to now.

MATERIAL AND METHODS

Sample

The anionic polysaccharide from *Porphyridium sp.* was prepared under as mild conditions as possible by growing *Porphyridium sp* at appropriate nutrient conditions in 1000-mL tubes. At the stationary state of growth, the cells were separated from the growth medium by centrifugation at 10,000 rpm for 20 min. The supernatant was dialyzed in Visking size 8 32/32 in. dialysis tubing (Medicell International Ldt., London, UK) against distilled water for 72 h and then against double-distilled water for 24–48 h at 4°C. In each case water was changed twice daily. The remaining solution was freeze-dried to obtain the water-soluble anionic polysaccharide as a white powder. Approximately 63% of total carbohydrates was found in this powder accompanied by ash (≈ 9%), protein (≈ 8%), sulfate (≈ 8%), glucuronic acid (≈ 8%) and unidentified compounds (≈ 4%). Constituting sugar monomers for the carbohydrate moiety consisted of glucose (≈ 15%), galactose (≈ 30%), mannose (≈ 1%), xylose (≈ 40%), rhamnose (≈ 0.1%), arabinose (≈ 0.8%), methylpentose (≈ 1.2%), methylgalactose (≈ 1.5%) and dimethylhexose (≈ 3.0%). The disaccharide 3-O→(αD-glucopyranosyl uronic acid)-L-galactopyranose has been found to be an important constituting unit within the isolated polysaccharide [10,11]. Guar was a commercially available guar-gum (Meypro-guar, Meyhall Chemical AG, Kreuzlingen, Switzerland). The utilized dextrans are from a commercially available standards kit (Pharmacosmos A/S, Viby Sj., Denmark). The hydroxyethylcellulose sample was commercially available (Natrosol 250 H Pharm, Hercules, MA, USA) (Table I).

For physicochemical characterization by means of rheology and size exclusion chromatography equipped with differential index detector and a low-angle laser light scattering instrument (SEC-DRI/LALLS), the white powder was dissolved in 0.05 M $NaCl_{aq}$ at room temperature yielding a turbid solution which was clarified by centrifugation with final concentrations between 0.08–0.1 mg/mL. With these concentrations reproducibility of DRI areas within ±3% could be achieved for repeated separations over one week with the SEC-DRI/LALLS-system. The separated sample components passed through an in-line membrane filter (cellulose acetate) with 0.45-μm pore size without increasing back-pressure, no significant differences with 0.65-μm pore-size filters were observed. In-line filters with 0.2-μm pore-size caused increasing back-pressure for hydroxyethylcellulose and the anionic polysaccharide after repeated injections and therefore have been judged not to be suitable. With DRI areas of dextran samples as references, recovery close to 100% of injected mass of the anionic polysaccharide was found.

Rheology

Static rheological experiments have been performed with a low-shear rheometer (Rheomat 30-Low Shear 2/100, Physica, Stuttgart, Germany) to determine the flow curve for the aqueous dissolved anionic polysaccharide in the range of shear rates between 10^{-2}–10^{2} s^{-1}. Dynamic rheological experiments were performed to determine the elastic properties have by means of an Oscillating Capillary Rheo- and Densitometer (OCR, Anton Paar, Graz, Austria). All rheological experiments were performed at 20°C.

TABLE I

Composition of different polysaccharide and "analogous factor" (see text) for the packing density of the different samples with the packing density of anionic-polysaccharide coils. The analogous factor was calculated as the ratio of absolute determined $M_w$abs = 8.4 × 10^6 g/mol and $M_w$cal which is achieved with guar, hydroxyethylcellulose, and dextran calibrations, respectively.

Sample	Abbr.	Constituting Monomers/ Linkages	Analogous Factor
anionic polysaccharide	A	glucose (≈15%), galactose (≈30%), mannose (≈1%), xylose (≈40%), rhamnose (≈0.1%), arabinose (≈0.8%) glucuronic acid (≈8%), β(1→3)-galactose and β(1→4) -3,6-anhydrogalactose with different degrees of SO_4^2 and -CH_3	
guar	G	β(1→4) linked mannose chain; α(1→6) linked galactose branches;	0.18
hydroxyethyl-cellulose	C	β(1→4) linked glucose chain; hydroxyethyl residues linked at the 2- and/or 3- and/or 6-position	1.28
dextran	D	α(1→6) linked glucose chain; α(1→4) and α(1→3) linked glucose-chain branches	1.05

Size-exclusion chromatography—Differential Refractive Index/Low-angle Laser Light Scattering (SEC-DRI/LALLS)

The separation of polysaccharide components by means of size-exclusion chromatography (SEC) with simultaneous dual detection of refractive index and scattering intensity was performed utilizing a set of TSK columns (guard-column PWH: 75 mm, 7-mm i.d.; GMPW, G6000PW: both 300 mm, 7-mm i.d. from ToyoSoda, Tokyo, Japan) combined with Separon HEMA BIO 1000 + 300: both 250 mm, 8mm i.d. from Tessek Ltd. Prague, Czekia) in series. The poly(hydroxylated hydroxyethyl methacrylate) (HEMA) particles of Separon HEMA BIO columns showed better resolution than TSK-columns in the molecular weight range below 200,000 g/mol. Since they can withstand pressure up to 30 MPa, these gels were utilized to shift accompanying low-molecular materials to high retention volumina. The particle size of HEMA BIO is 10 μm and has a nominal exclusion limits of 800,000–2,000,000 g/mol (HEMA BIO 1000) and 250,000–600,000 g/mol (HEMA BIO 300). The eluent was 0.05 M NaCl, an interferometric refractometer Optilab 903 at λ_{630} (Wyatt Technology, US) and a low-angle laser-light scattering instrument KMX-6 (LDC-Analytical, US) were employed for detection [12]. Sample volumes of 200 μL were injected and separated at a flow rate of 0.6 mL/min. Specific refractive index increment (s.r.i.i., *dn/dc*) for the samples have been determined by means of a interferometeric differential refractometer (Optilab 903, Wyatt Technology, USA) at a wavelength of 630 nm in aqueous 0.05 M NaCl. Within experimental error of ±3%, dextran $dn/dc_{630\,nm}$ = 0.152 mL/g, guar $dn/dc_{630\,nm}$ = 0.155 mL/g, hydroxyethylcellulose $dn/dc_{630\,nm}$ = 0.141 mL/g, and the anionic polysaccharide $dn/dc_{630\,nm}$ = 0.150 mL/g.

Universal calibration

Absolute molecular weights and intrinsic viscosity data for the investigated dextran-standards were utilized to establish universal calibration: $\log(M\,[\eta])$ versus retention volume). Assuming the occupied volume of polymer coils in solution as sphere equivalent V_e and taking the compressing forces on non-Newtonian polymer materials in SEC into consideration, a sphere-equivalent particle radius R_e can be computed according Equations (1) and (2). Sphere-equivalent radii of SEC-eluted non-Newtonian polymer coils are supposed to be smaller than the hydrodynamic radii R_H determined from translational diffusion coefficient D_T without applied compressing forces. For the case of Newtonian-type polymers, V_e turns into V_H and consequently R_e into R_H.

$$V_e = \frac{K\,M^{a+1}}{2.5\,N_A} \tag{1}$$

$$R_e = \sqrt[3]{\frac{3V_e}{4\pi}} \tag{2}$$

RESULTS

The 0.05 M $NaCl_{aq}$ dissolved anionic polysaccharide was separated on the described series of SEC-columns and if the DRI-elution profile is taken as criterion, an acceptable separation was achieved (Fig. 1). However, molecular data obtained from the LALLS detector which gave a weight average molecular weight M_W of 8.4×10^6 g/mol indicated no molecular separation: the obtained absolute calibration function ($\log M$ versus V_{ret}), established with DRI/LALLS-data suggested increasing molecular weight at increasing retention volumes (Fig. 1). Thus, if a separation was achieved, then separation of sample components according to the general SEC-separation mechanism was obtained, that is, separation according decreasing particle dimensions of eluted components with increasing retention volume V_{ret}.

Rheology was applied to investigate structural and/or interactive properties which could be responsible for the observed SEC separation characteristics. There, the anionic polysaccharide was found to be a non-Newtonian system showing strong dependence of viscosity on applied shear stress (Fig. 2). More detailed information was obtained by determining the elastic properties of the polymer/solvent-system, in particular the storage modulus G' correlated with reversible changes in particle geometry upon applied energy, and the loss modulus G'' correlated with irreversible destruction of molecular/supramolecular structures and simultaneous dissipation of energy. The ratio of these moduli, G'/G'', is a sensitive indicator for the magnitude of polymer/polymer interaction; values exceeding 1.0, which have been found for the aqueous-dissolved anionic polysaccharide, indicate pronounced elasticity of the system. This typically can be correlated with prominent polymer/polymer interaction and/or the existence of a (weak) gel (Fig. 3).

Another indication for particle/particle interaction would be c^*, the limiting concentration for overlapping of polymer coils. As a first approximation, c^* equals the reciprocal of the intrinsic viscosity and rough extrapolation of viscosity data from the flow-curve suggests an intrinsic viscosity of approximately 2×10^4 mL/g, and thus a c^* of ≈ 0.05

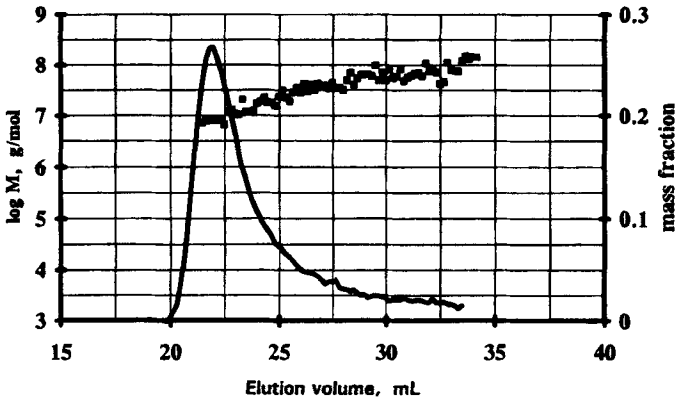

FIGURE 1 SEC chromatogram from the DRI detector of the investigated aqueous-dissolved anionic polysaccharide superimposed with the absolute molecular weights (■■■■) derived from the scattering intensity.

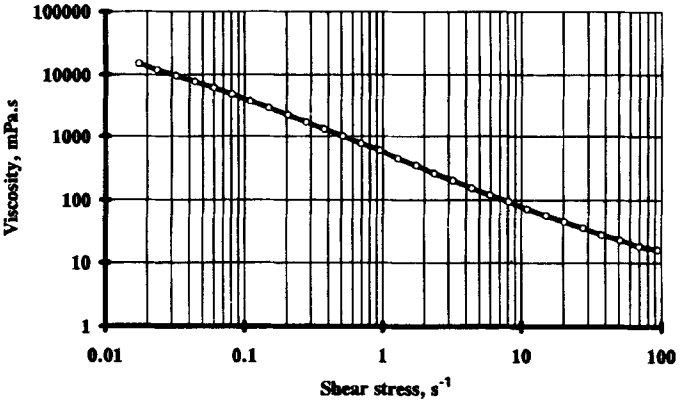

FIGURE 2 Rheological flow-curve of the aqueous-dissolved anionic polysaccharide.

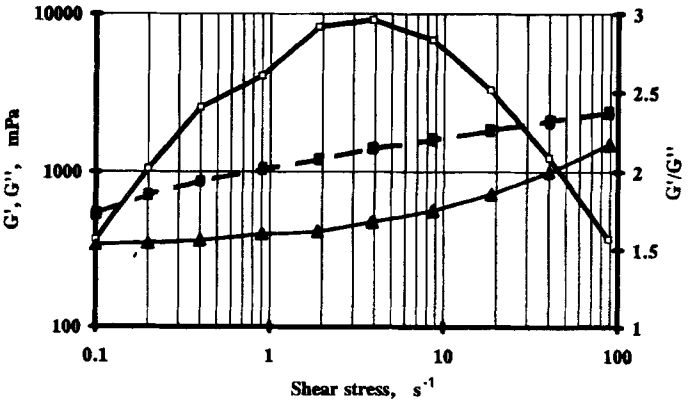

FIGURE 3 Elasticity of the anionic polysaccharide: storage modulus (G':-■-■-), loss modulus (G'':-▲-▲-), ratio G'/G'' (-□-□-);

mg/mL. The actually utilized bulk concentration for SEC separation was in the range of 0.08–0.1 mg/mL and therefore higher than c^*; however, due to dilution during the separation process, the monitored concentrations eluted from SEC were far below 0.05 mL/g. Although no overlapping should be expected, the observed high elasticity gives contrary information. Chemical composition (Table I) can explain this fact at least to a certain extent by an increased radius of interaction of charged (sulfated, uronylated) particles due to Coulomb forces and $\beta(1\rightarrow3)$- and $\beta(1\rightarrow4)$-glycosidic linkages which favor helical and β-sheet structures, respectively. Branching characteristics and hydrogen-bonding, for sure, indroduce additional possibilities for intra- and intermolecular interactions. Therefore, even below the overlap concentration, considerable elasticity for such polymers might be no real surprise.

To estimate the occupied volume of the anionic polymer particles, universal calibration was established with dextran standards. Additionally, three aqueous dissolved polysaccharides, (dextran (*D*), guar (*G*), hydroxyethylcellulose (*C*)) were investigated as reference materials to obtain more information about the correlation of coil dimensions and chemical polysaccharide characteristics, with special focus on changes for different glycosidic linkages (Table I). For these reference polysaccharides with Newtonian behavior in low-shear experiments, absolute calibration, log M versus V_{rel}, with SEC-DRI/LALLS data (Fig. 4) and transformation coefficients of absolute calibration into universal calibration were determined. These coefficients are related to the occupied volume of a polymer coil of a given molecular weight. Then, for the computation of eluted particle dimensions, transformation coefficients, even for the anionic polysaccharide into universal calibration, have to be determined.

As molecular weight calibration of the anionic polysaccharide cannot be transformed directely into universal calibration, the reference polysaccharide calibrations were tested for their suitability to re-achieve the absolutely determined weight average molecular weight (M_W) for the SEC data of anionic polysaccharide. The higher the similarity of achieved M_W by applying the reference calibration to the chromatogram of the anionic polysaccharide, the higher the similarity of polymer-coil packing density can be assumed.

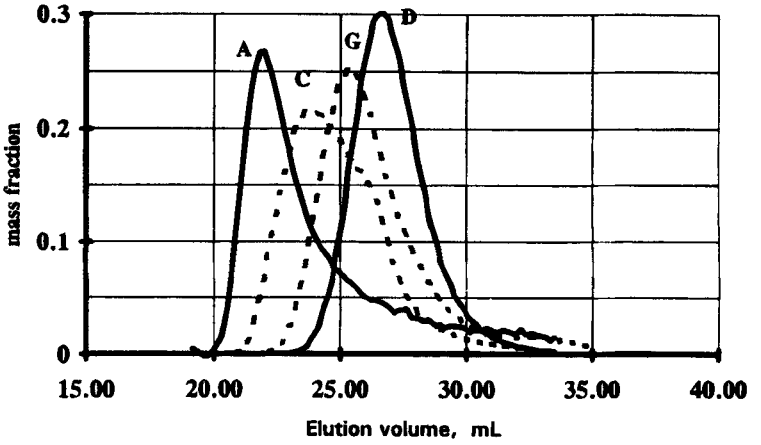

FIGURE 4 SEC chromatograms of anionic polysaccharide (*A*), hydroxyethylcellulose (*C*), guar (*G*), and dextran (*D*)

As an indicator for the degree of similarity an "analogous factor" was computed as the ratio of the initially absolute determined M_W for the anionic polysaccharide and the M_W which is achieved applying reference polysaccharide (D, C and G) calibration. An analogous factor of 1.0 stands for identical packing density between the anionic and reference polysaccharides; values higher than 1.0 indicate more compact coils for the anionic than for the reference polysaccharides; and values smaller than 1.0 the opposite situation. The resulting analogous factors are listed in Table I and show that the coils of anionic polysaccharide are definitely more compact than those of hydroxyethylcellulose, significantely more loose packed than those of guar, and close to the dextran. The resulting dextran-analogous calibration function, containing information about the correlation of occupied volume by a polymer coil with dextran-like packing characteristics at a certain retention volume, then was transformed into universal calibration (Fig. 5) yielding transformation coefficients K and a. Finally, with Equations (1) and (2) the occupied sphere equivalent volume V_e and the sphere equivalent radius R_e of eluted polysaccharide components for the retention volumes were computed. The resulting mass distribution of sphere-equivalent radii is shown in Figure 6 and ranges between ≈2–140 nm with a maximum of particle mass around 90 nm (Table II).

CONCLUSION

The investigated anionic polysaccharide is a heteropolymer with a number of neutral and charged, namely uronylated and sulfated, monomers. Absolute molecular weight for the anionic polysaccharide was determined by means of SEC-DRI-LALLS. An overlap concentration was estimated from data of low shear experiments with ≈0.05 mg/mL and high elasticity. But even with significantely lower concentrations, no SEC separation according to decreasing molecular weights could be found. Nevertheless, the elution profile suggests

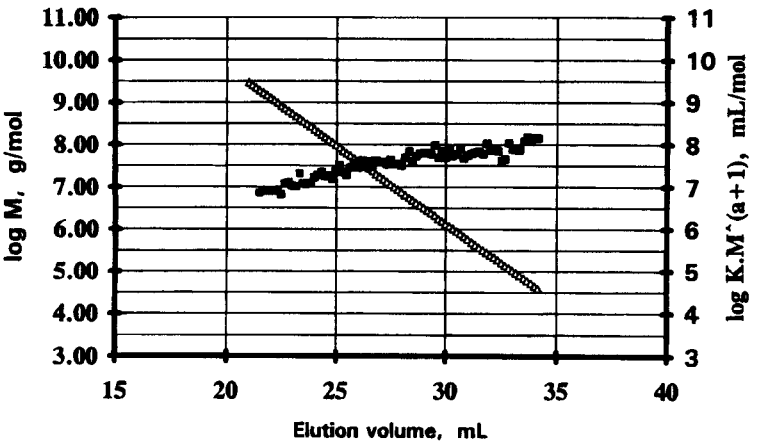

FIGURE 5 Universal calibration established with dextran standards (with absolute molecular weights from SEC-DRI/LALLS data and with intrinsic viscosity data) (ΔΔΔ) and absolute molecular weight of SEC-eluted components of anionic polysaccharide (■■■).

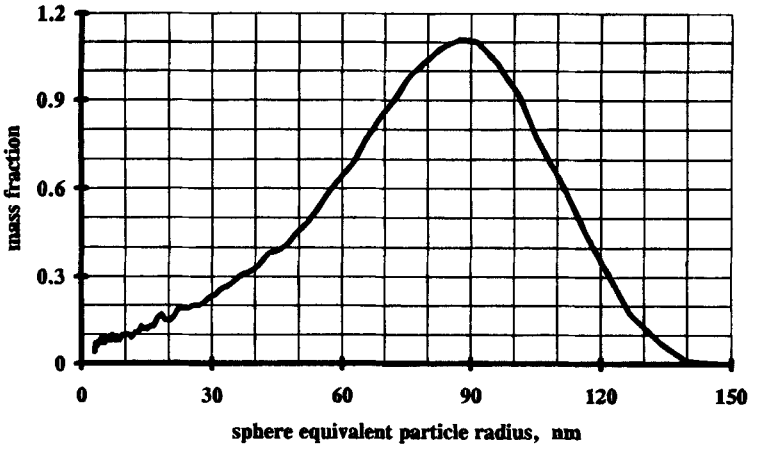

FIGURE 6 Distribution of polymer coil dimensions for the SEC-separated anionic polysaccharide components in terms of sphere-equivalent radii distribution.

TABLE II

Physicochemical characteristics of the investigated anionic polysaccharide.

Parameter	Result
investigated sample concentration (bulk)	0.08–0.1 mg/mL
solvent	0.05 M NaCl
temperature for dissolution, rheological and SEC-DRI/LALLS experiments	20°C
weight average molecular weight M_w	8.4×10^6 g/mol
estimated overlap concentration c^*	≈0.05 mg/mL
viscosity η for shear rates $10^{-2} \rightarrow 10^2\ s^{-1}$	$10^{4.1} \rightarrow 10^{1.1}$ mPa s
storage modulus G′ for shear rates $10^{-2} \rightarrow 10^2\ s^{-1}$	$10^{2.7} \rightarrow 10^{3.2}$
ratio G′/G″ for shear rates $10^{-2} \rightarrow 10^2\ s^{-1}$	1.5–3
dimensions for SEC-eluted components in terms of sphere-equivalent radius $\mathbf{R_e}$:	
range: R_eD	2–140 nm
maximum of mass: R_e(Max)	90 nm

separation of sample components according decreasing particle dimensions. Additional investigation of three hydrodynamically differing polysaccharides as reference materials and introduction of universal calibration enabled an estimation of particle size distribution for the anionic polysaccharide in terms of sphere-equivalent radii. The high elasticity of the anionic polysaccharide should be due to intra- and intermolecular interactions based on the chemistry, including compressibility of the polymer coils upon applied forces. Therefore, the determined occupied volume of the eluted components is not identical to the hydrodynamic volume in a relaxed state but smaller. In addition, intra- and interactive properties such as branching characteristics, will influence the final occupied volume and thus, the position of SEC elution: for a polymer coil with a given molecular weight, the occupied volume will be smaller with high compression and higher degree of branching. This information is not accessible by single mass detection (e.g., DRI) connected to SEC

separation, but by simultaneous detection of low angle scattering intensity (LALLS), only, because LALLS responds to molecular weight of eluted components independent of their time of elution from the chromatographic system. But, as a matter of fact, complimentary information would be provided by additional detection of viscosity, especially for studying branching characteristics.

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