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Concentration regimes of solutions of levan polysaccharide from *Bacillus* sp.

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Abstract

The rheological properties of aqueous solutions of levan polysaccharide from *Bacillus* sp. were studied at 20.0 °C over a wide range of concentrations. At low concentrations the solutions were Newtonian, becoming shear thinning at higher concentrations. The intrinsic viscosity of this levan was found to be 0.14 dL/g, which is close to that of other levans. However, whereas other polysaccharides exhibit up to three concentrations regimes (dilute, semi-dilute, and concentrated) in a double logarithmic plot of the specific viscosity versus space occupancy, this levan exhibits four distinct concentration regions, at least three of which are linear. To the best of our knowledge, this is the first report of a solution of any kind exhibiting more than three distinct regions in this type of plot.
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Keywords: Levan; Polysaccharide; Intrinsic viscosity; Specific viscosity; Space occupancy; *Bacillus sp.*; Coil overlap parameter; Critical concentration; Dilute regime; Semi-dilute regime; Concentrated regime; Concentration regimes

1. Introduction

Levan is one of two main types of fructans, which are naturally occurring homopolymers of fructose. Its main chain is composed of repeating five-member fructofuranosyl rings connected by β(2→6) links (Fig. 1a). Branching of the main chain results when fructofuranosyl rings connect through β(2→1) linkages. In contrast, in inulin, the other main type of fructan, the main chain is formed by β(2→1) linkages (Fig. 1b). Levans produced by different organisms differ in their molecular weight and degree of branching. Levans from plants generally have molecular weights ranging from about 2000 to 33,000 Da (Rhee et al., 2002). Bacterial levans are much larger than those produced by plants, with multiple branches and molecular weights ranging from 2 to 100 million Da (Keith et al., 1991).

Much of the work on fructans has centered on inulin because of its tendency to form gels in aqueous solution, which makes inulin useful for replacing fats in food products while retaining product texture. Levan, on the other hand, is non-gelling and is generally considered non-swelling in water at room temperature (Kasapis & Morris, 1994; Stivala & Bahary, 1978). Many past studies on levan were motivated by its presence in dental caries (Ehrlich et al., 1975; Newbrun & Baker, 1968), although more recent works concentrate on its role in plant pathogenesis (Kasapis & Morris, 1994; Kasapis, Morris, Gross, & Rudolph, 1994), its antitumor properties (Calazans, Lima, deFranca, & Lopes, 2000; Calazans, Lopes, Lima, & deFranca, 1997; Yoo, Yoon, Cha, & Lee, 2004), its cholesterol-lowering properties (Yamamoto et al., 1999), and its use as an environmentally benign adhesive (Combie, Steel, & Sweitzer, 2004).

Levan is an unusual polysaccharide in that it has a relatively low intrinsic viscosity compared to other molecules of similarly high molecular weights. Intrinsic viscosity $[\eta]$ is defined as

$$[\eta] = \lim_{c\to 0} \frac{\eta_r - 1}{c} = \lim_{c\to 0} \frac{\eta_{sp}}{c} \quad (1)$$

where η_r, the relative viscosity, is ratio of the viscosity of the solution η to the viscosity of the pure solvent η_0; η_{sp}, the specific viscosity, is defined as $\eta_r - 1$; and c is the concentration of polymer in solution, usually in deciliters per gram. Previous studies have found intrinsic viscosities for levan in water ranging from 0.07 to 0.18 dL/g for levans with molecular weights ranging from 16 to 24 million Da (Ehrlich et al., 1975; Newbrun & Baker, 1968; Stivala & Bahary, 1978). Typical commercial polysaccharides such as cellulose, carrageenan, xanthan, and guar gum have intrinsic viscosities in the range of 5–50 dL/g (Carriere, Amis, Schrag, & Ferry, 1993; Chronakis, Doublier, & Piculell, 2000; Cuvelier & Launay, 1986; Funami et al., 2005; Jumel et al., 1996;

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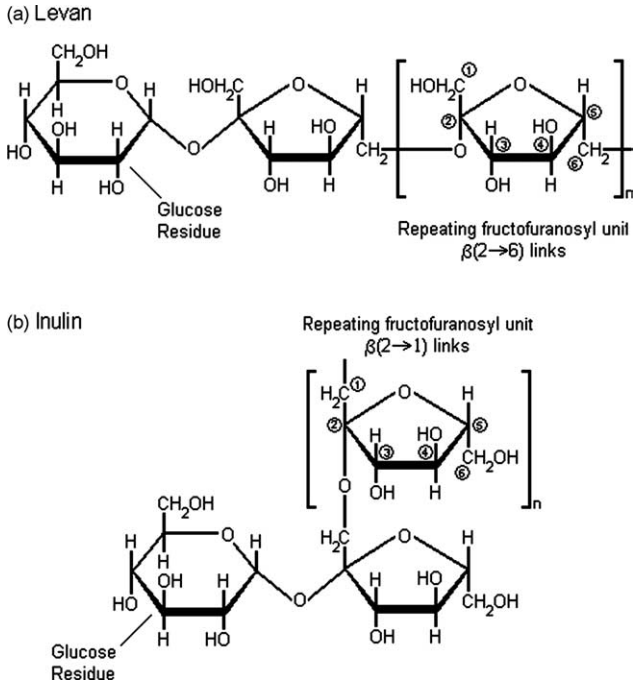

Fig. 1. Chemical structure of the two main types of fructans: (a) levan and (b) inulin.

Nickerson, Paulson, & Hallet, 2004). Flexible chain polysaccharides such as dextrose and compact coil polysaccharides such as amylose have intrinsic viscosities of about 1 dL/g (Kasapis & Morris, 1994).

Low intrinsic viscosity at high molecular weight implies a compact spherical shape. Electron microscopy indicates that the molecular shape of levan is ellipsoidal (Newbrun, Lacy, & Christie, 1971), which suggests that the branches extend radially and are formed at approximately the same rate (Rhee et al., 2002). Furthermore, the intrinsic viscosities of levans are comparable to those of spherical and dendritic polymers, which typically exhibit intrinsic viscosities in the range of 0.05–0.10 dL/g (Striolo, Prausnitz, Bertucco, Kee, & Gauthier, 2001).

Dilute solution behavior occurs when the concentration of a solute is low enough that the solute molecules behave as if they are alone in a vast sea of solvent. Concentrated solution behavior occurs when solute molecules interact physically with other solute molecules, while semi-dilute solution behavior occurs when solute molecules are indirectly affected by the presence of other solute molecules. The relationship between viscosity and the space occupancy (the product of concentration, c, and intrinsic viscosity, $[\eta]$) has been studied for many polymer–solvent systems to determine the concentrations at which the solutions transition between the dilute, semi-dilute, and concentrated regimes. A double logarithmic plot of specific viscosity, η_{sp}, versus the space occupancy, $c[\eta]$, of cellulose shows one linear region and no transitions for space occupancies between 1.78 and 31.6 (Jumel et al., 1996). Most disordered polysaccharides exhibit two distinct linear regions with an abrupt change from one to the other on this type of plot (Morris, Cutler, Ross-Murphey, Rees, & Price, 1981). The first linear region has a slope of 1.4, which shifts to a slope of 3.3 at a space occupancy of 4 (critical concentration $c^*=4/[\eta]$). Kasapis et al. (1994) found that for levan from *Pseudomonas syringae* pv. *phaseolicola*, the double-logarithmic plot described above resulted in three linear regions with abrupt changes at the critical concentrations $c^*=0.75/[\eta]$ and $c^{**}=3.6/[\eta]$.

For the successful application of levan in foods and adhesives it is critical to understand the relationship between molecular weight and solution viscosity and to determine the critical concentrations at which the solution behavior changes between the dilute, semi-dilute, and concentrated regimes. The aim of the present work is to investigate the rheological properties of levan produced from *Bacillus sp*. in order to determine the critical concentrations of levan–water solutions.

2. Materials and experimental methods

2.1. Materials

Aqueous solutions were prepared from samples of levan polysaccharide from *Bacillus* sp. obtained from Montana Biotech SE, Inc., (Rock Hill, SC). Solution concentrations ranged from 0.05 to 60% based on the weight of undried levan. The moisture content in the levan samples was determined by drying a sample of this levan at 55 °C over a period of 1 year. It was found to contain 7.91% water. Undried levan was used to prepare the samples but the moisture content was taken into account when calculating the concentration of levan in the solutions. Solution measurements at low polymer concentrations were used to determine the intrinsic viscosity, $[\eta]$, and the value obtained for the intrinsic viscosity was then used to prepare a logarithmic plot of η_{sp}/c versus $c[\eta]$ for a large range of concentrations.

Solution densities were measured at 20 °C using a Paar Density Meter model DMA 48 (Anton Paar, Ashland, VA). The kinematic viscosity (the ratio of viscosity to density) of the dilute solutions was measured using two Cannon-Fenske capillary viscometers (Cannon Instrument Company, State College, PA) immersed in a constant temperature bath held at 20.0 °C. The product of the kinematic viscosity and the density was used to calculate the viscosity of the dilute solutions. The viscosity of the more concentrated solutions was measured directly as a function of shear rate in a Rheometrics Fluids Spectrometer RFS II (Rheometrics Scientific, Piscataway, NJ). Measurements were performed in a Couette device kept near 20 °C at shear rates mostly ranging from 0.5 to 500 s^{-1}.

2.2. Determining critical concentrations

Often, the viscosity of dilute solutions of a polymer can be fit to the Huggins equation

$$\frac{\eta_{sp}}{c} = [\eta] + k_H[\eta]^2 c \quad (2)$$

where k_H is the Huggins coefficient. A solution is generally considered dilute if its viscosity is less than twice the viscosity of the pure solvent. When the Huggins equation is applicable, a plot of η_{sp}/c versus c results in a straight line with y-intercept equal to $[\eta]$ and slope equal to $k_H[\eta]^2$. Typically, for polymers with good solvents, $k_H = 0.4 \pm 0.1$ (Rodriguez, Cohen, Ober, & Archer, 2003). As will be explained below, the intrinsic viscosity, $[\eta]$, is related to the hydrodynamic volume occupied by a unit mass of the polymer in dilute solution, and the product $c[\eta]$, called the space occupancy or the coil overlap parameter, is related to the effective volume fraction of the polymer in solution.

2.3. Finding molecular weight from viscosity

For a polymer solution at very low concentrations:

$$\frac{\eta}{\eta_0} = 1 + [\eta]c \quad (3)$$

Einstein (1906) found that for an infinitely dilute suspension of hard spheres

$$\frac{\eta}{\eta_0} = 1 + \frac{5}{2}\phi \quad (4)$$

where ϕ is the volume fraction of spheres in the suspension. Thus, if we can assume that the polymer molecules behave hydrodynamically as solid spheres

$$[\eta]c = \frac{5}{2}\phi \quad (5)$$

and the product $[\eta]c$ would be proportional to the volume fraction of the polymer in solution. This relationship leads to

$$[\eta] = \frac{10\pi}{3} N_A \frac{R_H^3}{M} \quad (6)$$

where N_A is Avogadro's number and M is the molecular weight of the polymer. Thus, viscosity data can be used as a sensitive measure of the molecular weight of a polymer if the hydrodynamic radius is accurately known.

3. Results and discussion

3.1. Determining critical concentrations

The Huggins plot shown below (Fig. 2), for solutions of *Bacillus* sp. levan with concentrations less than 6 g/dL, is equivalent to Fig. 1 in Kasapis et al. (1994). The y-intercept of this plot is the intrinsic viscosity $[\eta]$. For the levan from Montana Biotech we find $[\eta] \approx 0.14$ dL/g. Kasapis et al. (1994) report $[\eta] \approx 0.17$ dL/g for levan from *P. syringae* pv. *phaseolicola*. Typical values for the intrinsic viscosity of linear polymers are at least an order of magnitude larger. From the Huggins plot, the Huggins coefficient was $k_H \approx 0.59$, which is somewhat larger than that of many polymers in good solvents.

In general, entangled polysaccharide networks exhibit a shear-thinning behavior that is linear for plots of η versus $\eta\gamma^{0.76}$ (Morris, 1990). As shown in Fig. 3, levan solutions remained Newtonian (viscosity independent of shear rate) until concentrations up to about 30 mass%. The solutions were shear-thinning at higher concentrations. A plot of η versus $\eta\gamma^{0.76}$ for a 60 mass% solution of the *Bacillus* sp. levan under investigation (55.3 mass% dry levan), seen in Fig. 4, does not show the described linearity despite the presence of shear thinning, indicating that molecular interactions in *Bacillus sp*. levan may include but are not limited to physical entanglements. In contrast, the levan from *P. syringae* pv. p*haseolicola* studied by Kasapis & Morris (1994) did show the described linearity of shear-thinning at 26.5% w/v.

Fig. 5 is a double logarithmic plot of the specific viscosity, η_{sp}, versus space occupancy, $c[\eta]$, for the levan sample from Montana Biotech's *Bacillus* sp. This plot reveals four distinct regions, at least three of which are linear. To the best of our knowledge, this is the first report of a solution

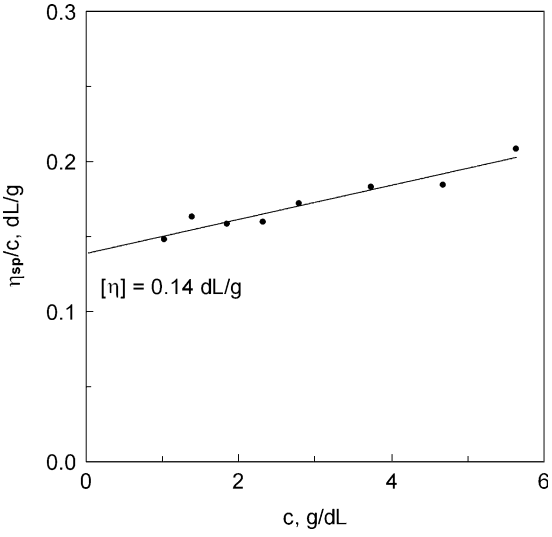

Fig. 2. Huggins plot for *Bacillus* sp. levan at 20 °C.

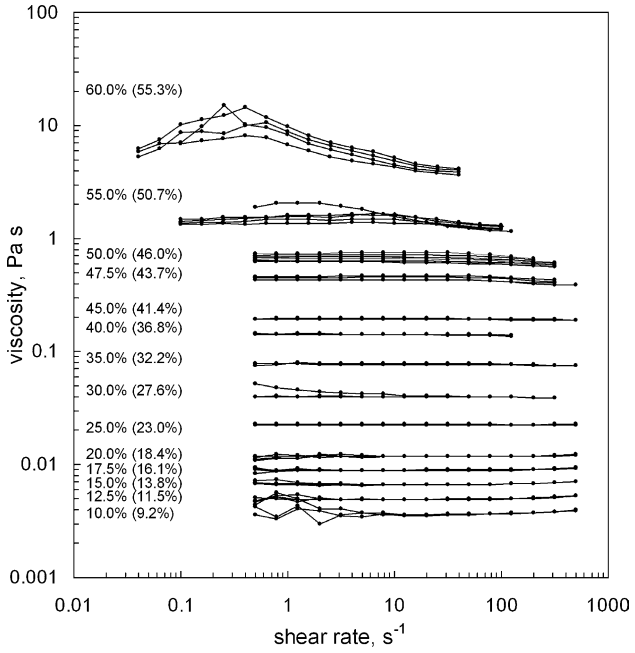

Fig. 3. Viscosity of aqueous levan solutions at 20 °C. Concentration is shown in mass percent of undried levan, with concentrations of dry levan in parenthesis. Shear thinning is seen at concentrations above 30 mass% undried levan.

exhibiting more than three distinct regions in this type of plot. The low-concentration region has a slope of 1.2 up to a space occupancy $c[\eta]$ of 1.1. The medium-concentration region has a slope of 2.0 for $c[\eta]$ between 1.1 and 3.1. At higher concentrations, a linear region with a slope of 3.6 and another region with a slope of approximately 9.7 can be discerned with the transition at a space occupancy of 7.0.

At moderate concentrations, when the viscosity was shear-rate dependent, the low shear limit of the viscosity was used to calculate η_{sp}. As Fig. 3 suggests, determining the low shear limit was difficult at the higher concentrations (55 and 60% levan) because the viscosity did not plateau, exhibiting a maximum instead. The value of the slope in the last region falls between 7.6 and 11.1 depending on which values from Fig. 3 are used as the low-shear limits of the viscosity. The reported slope of 9.7 for the last region was obtained using an intermediate value of the viscosity at low shear rates for the concentrated solutions.

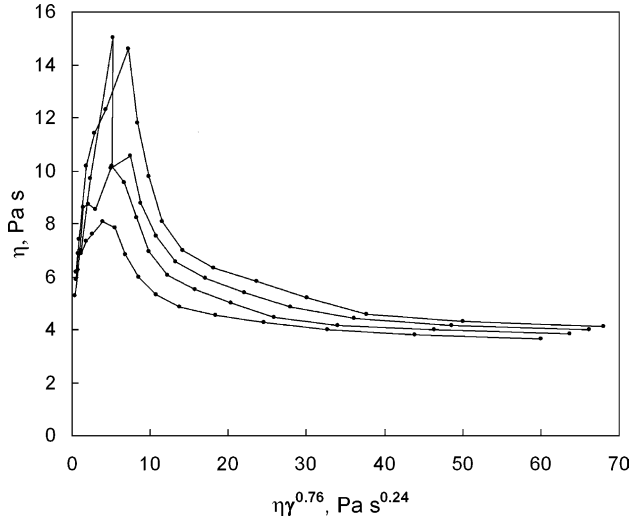

Fig. 4. Viscosity of 60 mass% undried levan (*Bacillus* sp.) at approximately 20 °C as a function of the product of viscosity and shear rate to the power 0.76. Viscosity is in Pascal second and shear rate in per second. Four runs are plotted, and viscosity increased with each run.

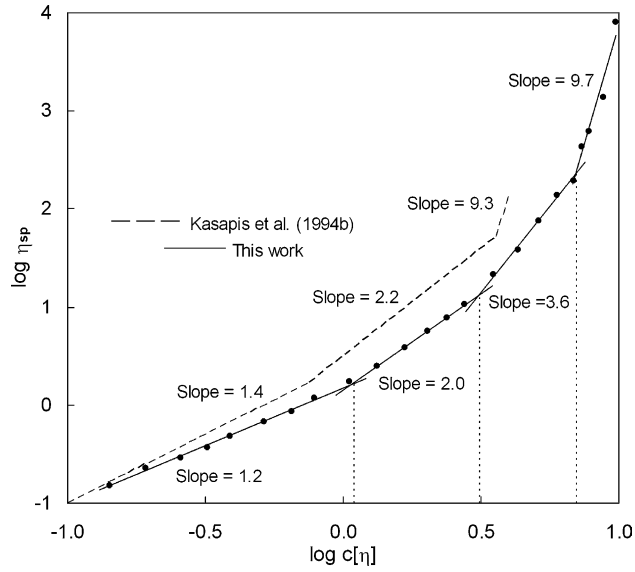

Fig. 5. Concentration dependence of specific viscosity for levan at 20 °C. Solid lines are best fit to data points. Dashed line is reproduced from Kasapis et al. (1994). Vertical lines indicate critical concentrations.

Also shown in Fig. 5 is the fit to the data in Fig. 5 of Kasapis et al. (1994) for the solutions of their levan. They observed only three regions in their plot. The first region had a slope of 1.4 up to a space occupancy of 0.75. The second has a slope of 2.2 for space occupancies between 0.75 and 3.6. At space occupancies above 3.6, their plot had a slope of 9.3. At no space occupancy did they find a region with a slope comparable to that of our third region, which had a slope of 3.6.

Morris et al. (1981) find that for most disordered polysaccharide solutions this type of plot exhibits only two regions. The first region has a slope of 1.4 and the second region has a slope of 3.3 with the transition occurring at a space occupancies of 4. They also found several disordered polysaccharide solutions that diverge from the typical behavior, exhibiting an earlier transition and a steeper slope in the second region. In these systems gels or precipitates formed under certain conditions. The formation of junctions between stiff, structurally regular chain sequences in addition to physical entanglements was used to explain the 'enhanced viscosity' of the materials. Newlin, Lovell, Saunders, and Ferry (1962) report that synthetic polymers systems of poly(*n*-butyl methacrylate) show a similar steep dependence of viscosity on concentration and explain the behavior in terms of long-range intermolecular coupling or hyperentanglement.

Morris et al. (1981) consider polymer chain length and chain stiffness as two of the main factors contributing to the magnitude of $[\eta]$. They argue that steric crowding of most

polysaccharides limit the possible range of orientations, leading to decreased chain flexibility and higher solution viscosities at relatively lower concentrations than comparable non-carbohydrate polymers. They point out the polysaccharide dextran as an exception, with the relatively low intrinsic viscosity of $[\eta] = 0.23$ dL/g (Uraz & Güner, 1997) which they attribute to the additional conformational freedom by rotation about dextran's C(5)–C(6) bond. As a result, dextran shows much lower solution viscosities that most other polysaccharides, and the transition from dilute to concentrated behavior appears less pronounced than for other polymer chains. Unlike dextran, the linkages in levan are through oxygen atoms that are connected directly to the sugar ring, which would make the links less flexible. However, levan is composed of D-fructofuranose rings that are more flexible than the D-glucopyranose rings of dextran (French, 1988). The increased flexibility of the rings may explain why levans have an even lower intrinsic viscosity than dextran.

The levans from *Bacillus* sp. (this work) and from *P. syringae* pv. *phaseolicola* (Kasapis et al., 1994) have different intrinsic viscosities, critical concentrations, and number of transitions. The lower intrinsic viscosity and the more gradual transition from dilute to concentrated behavior of the *Bacillus sp*. levan may indicate that its chains are somewhat more conformationally flexible than those of the levan from *P. syringae* pv. *phaseolicola*. Differences in branching between the two types of levans may also contribute to the difference in their flexibility. Branching will also have a direct effect on resistance to flow (viscosity) by making it more difficult for individual molecules to move (reptate) through the entangled network of surrounding chains.

As described stated Section 2.2, solutions are generally considered dilute when their viscosity is less than about twice that of the pure solvent. The first transition occurs at a viscosity 2.7 times that of the pure solvent, and is thus attributed to the onset of semi-diluted solution behavior. Following the second transition, the slope is 3.3, which is similar to the expected slope of 3.6 for most disordered polysaccharides in their concentrated regime (Morris et al., 1981). Therefore, the second transition is assigned to the onset of concentrated behavior of levan molecules in solution. The onset of a very steep slope following the third transition may indicate that hyperentanglements took place as well as other interactions between molecules. This conclusion is further supported by the plot shown in Fig. 4 that indicated molecular interactions may include but were not limited to physical entanglements.

3.2. Finding molecular weight from viscosity

Earlier experiments to determine the molecular weight of this levan utilized size exclusion chromatography and estimated the molecular weight to be 1–2 million Da (unpublished data, Montana Biotech SE, Inc.,). Due to the nature of their manufacture, some variation between samples of levan is expected. However, these experiments did not calibrate the column with a similar spherical polymer to take into account the apparent compact, spherical structure of levan and are thus suspect.

Levans of comparable intrinsic viscosity produced by other bacteria typically have molecular weights of 16–24 million Da (Ehrlich et al., 1975; Kasapis & Morris, 1994; Newbrun & Baker, 1968; Stivala & Bahary, 1978). Applying the theory outlined in Section 2.3 to this range of molecular weights, Eq. (6) predicts a hydrodynamic radius of about 33–38 nm for levan with an intrinsic viscosity of 0.14 dL/g. Ploehn (2004) carried out dynamic light scattering on Montana Biotech *Bacillus sp*. levan to determine the size of levan. Generally, only one peak was seen, and the peak intensity was located at a particle radius of about 95 nm. By Eq. (6), such a radius would result in a molecular weight of 386 million Da. Earlier light scattering experiments had produced peaks ranging from 25 to 46 nm. Both of these light scattering experiments was performed on a different batch of *Bacillus* sp. levan. Therefore, it is difficult to conclude whether the hard sphere theory applies to dilute solutions of this levan polysaccharide. Further light scattering experiments from the same batch would be necessary, and these would need to be compared to an additional method such as well calibrated size exclusion chromatography.

4. Conclusions

Rheological properties of dilute solutions of levan produced by *Bacillus* sp. were investigated. The intrinsic viscosity for this type of levan is comparable to that of levans produced by other bacteria. Viscosity data indicate that molecular interactions are not limited to physical entanglements as has been reported for another levan and shear-thinning behavior appears at a higher concentration than for other levans. A double logarithmic plot of the specific viscosity, η_{sp}, versus space occupancy, $c[\eta]$, reveals four distinct regions, at least three of which are linear. To the best of our knowledge, this is the first report of a solution exhibiting more than three distinct regions in this type of plot.

Intrinsic viscosity data were used to estimate the molecular size of levan samples. Because this polymer is considered to be a spherical macromolecule, the Einstein equation for hard spheres was used to predict the hydrodynamic radius based on the molecular weight range of levans with similar intrinsic viscosities. The hydrodynamic radii as determined by dynamic light scattering were too variable among samples and labs to confirm calculated values. Therefore, the applicability of the hard sphere theory for dilute solutions of this polymer could not be substantiated.

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