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Comparative Measurement of the Molecular Weight of an Antineoplastic Glucan from BCG Vaccine

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Abstract

Bacillus Calmette-Guérin (BCG) vaccine, developed originally for the prophylaxis of tuberculosis, is a potent immunostimulant used to treat superficial bladder carcinoma in man. The aim of this study was to compare the molecular weight and self-association properties of an antineoplastic glucan (PS1A1) extracted from BCG vaccine as determined by different techniques including diffusion, light-scattering and chromatographic methods.

In the diffusion experiments, a semi-empirical relationship was derived between the effective diffusion coefficients, D_p, and the weight-average molecular weights, M_W, of several dextrans used as standards, according to the equation $D_p = 2{\cdot}233 \times 10^{-6} \times M_W^{-0{\cdot}66}$. On the basis of this relationship, the molecular weight of PS1A1 was found to be 57·4 kDa, although, unexpectedly, membrane association was high, most probably because of molecular branching. In the light-scattering experiment it was observed that, unlike dextran, PS1A1 undergoes concentration-dependent multimerization in water. However, the molecular weight of PS1A1 in 0·1 M sodium chloride ranged from 60 to 68 kDa, with a mean of 65 kDa, over the same concentration range. This value was in agreement with the molecular weight determined for PS1A1 by gel-filtration chromatography in previous studies, suggesting that 65 kDa represents the approximate monomeric size of the unassociated molecule.

Thus, it was evident that the aggregation was suppressed by electrolyte. Elemental analysis by X-ray fluorescence showed that PS1A1 contained carbon, oxygen, hydrogen and phosphorus, indicating that hitherto unobserved ionized phosphate groups might promote electrostatic interactions.

Bacillus Calmette-Guérin (BCG) vaccine, a suspension of live attenuated *Mycobacterium bovis* cells, has been used for the prophylaxis of tuberculosis for over 70 years. The vaccine is a potent, non-specific immunostimulant and has antineoplastic activity against some tumours, including bladder cancer (Morales et al 1976; Lamm 1985, 1995). In 1990 BCG was approved by the United States Food and Drug Administration for treatment of superficial bladder carcinoma in man (Anon. 1990; Nightingale 1990); it is currently the preferred treatment for this condition. However, adverse effects, such as systemic mycobacteriosis (Steg et al 1992) and occasional enhancement of tumour growth (Klegerman et al 1991), have been attributed to the administration of whole, living bacteria.

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Recent investigations have been directed at isolating, identifying and characterizing the non-viable components responsible for the antineoplastic activity of BCG. Lou et al (1994) obtained an active boiling water extract of BCG (Tice substrain), termed PS1, containing at least 50% carbohydrate. Chromatography on Sephadex LH-20 and Sephadex G-75 columns led to fractionation of PS1 into at least six sub-fractions. The fraction with maximum activity in a murine S180 sarcoma model was denoted PS1A1, and was further characterized. Both gas chromatography and nuclear magnetic resonance (NMR) spectroscopy

showed PS1A1 to be a glucose homopolymer, or glucan, with predominantly α-(1 → 6) linkages (Wang et al 1995). In addition, α-(1 → 4)-linked side-chains were detected along the main chain, with a branch point every five to six residues.

The structures of PS1A1 and dextran are similar (Figure 1), although there are differences in the extent of branching of the two molecules. Dextrans produced by the B-512 strain of *Leuconostoc mesenteroides* are glucose polymers with 95% α-(1 → 6) linkages, the remaining 5% of monomers having α-(1 → 3)-linked glucose side-chains. Fewer than 15% of these branches were reported to be more than two monomers long (Larm et al 1971). Although there are differences in the extent of branching of dextrans and PS1A1, dextrans were considered to be reasonable comparative standards for characterization studies.

Several literature reports describe naturally occurring bacterial and fungal polysaccharides with immunomodulatory activity (Sasaki et al 1976; Whistler et al 1976; Lien 1990). These active glycans have different chemical structures and sugar compositions, but many are D-glucans. In general, soluble D-glucans have antitumour activity, particularly if they are linear, without excessively long branches; high molecular-weight D-glucans are usually more biologically active than those of low molar mass (Whistler et al 1976; Lien 1990).

The purpose of the work described in this paper was to compare the molecular weight of the antineoplastic glycan PS1A1 determined by different techniques including diffusion, light-scattering and chromatographic methods, in an attempt to define more clearly the effective molecular weight. Because most polysaccharides do not dissolve as monomers, this study has also evaluated the tendency of these materials to self-associate and determined possible mechanisms underlying such aggregation phenomena.

Materials and Methods

Chemicals

Dextran standards with weight-average molecular weights (M_W) 9·3 kDa, 19·5 kDa, 39·2 kDa and 73 kDa were purchased from Sigma (St Louis, MO). Analytical grade monobasic sodium phosphate monohydrate, anhydrous dibasic sodium phosphate, sodium chloride, phenol and concentrated sulphuric acid (96%) were obtained from Fisher Scientific (Itasca, IL). PS1A1 was extracted from the Tice substrain of BCG lot 105158-C (manufactured at the Institute for Tuberculosis Research in 1983 and stored at −20°C) by the boiling water process described by Lou et al (1994) and Wang et al (1995).

Diffusion technique

In a preliminary experiment the molecular weight of PS1A1 was determined by comparing the diffusion of PS1A1 through inert porous membranes with that of four commercial dextran standards. Microporous membranes with a pore size of 0·22 μm, a porosity of approximately 80% and a mean thickness of 150 μm, manufactured from mixed cellulose esters, were purchased from Micron Separations (Westborough, MA). The exposed membrane area was 0·666 cm^2. Experiments were performed using four Valia Chien side-by-side diffusion cells coupled with one-speed drive consoles (Crown Glass Company, Somerville,

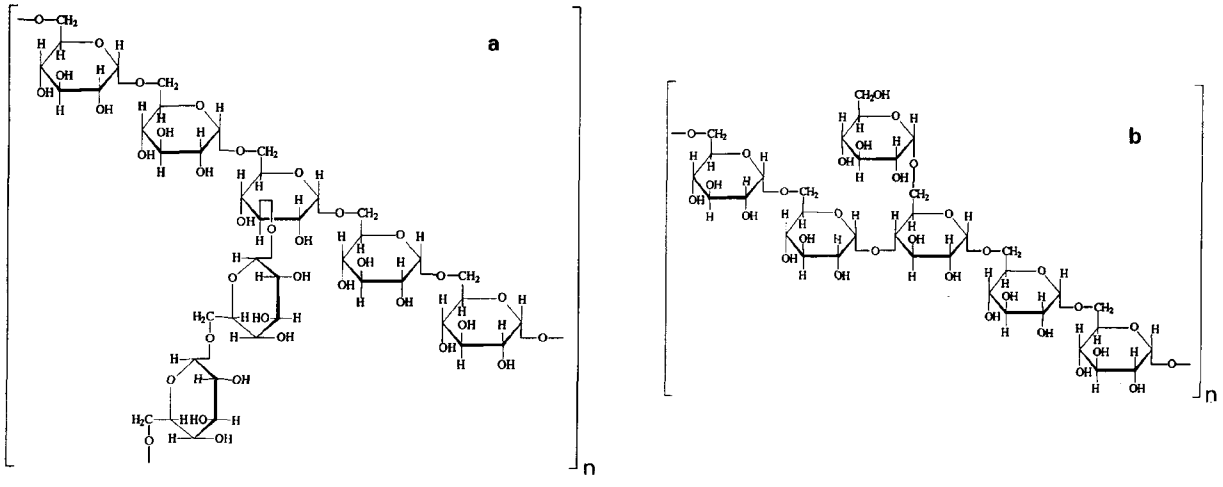

Figure 1. Repeating units of (a) dextran and (b) PS1A1. Both glucans have an α-(1 → 6)-linked main chain but the branch lengths and branch linkages are different (1 → 4 for PS1A1 and 1 → 3 for dextrans). The frequency of branching of the compounds is also different, although this is not evident in this figure.

NJ). Temperature control (26·0 ± 1·0°C) was maintained by a circulating water bath.

The donor compartment of each of the four diffusion cells was filled with a solution of dextran in water (1 mg mL^{-1}; 3·8 mL) and the receptor compartment was filled with an equal volume of water. The solutions in both the donor and receptor compartments were stirred (600 rev min^{-1}) to prevent boundary-layer resistance effects. Diffusion of dextran molecules through the membrane was periodically monitored by removing 100-μL samples from the chambers and measuring the total carbohydrate content by the phenol–sulphuric acid assay (Dubois et al 1956). This experiment was repeated using solutions (1 mg mL^{-1}) of dextran standards of weight-average molecular weight 9·3 kDa, 19·5 kDa, 39·2 kDa and 73·0 kDa and a solution (1 mg mL^{-1}) of PS1A1.

The data were analysed by use of the equation (Zentner et al 1979; Bohrer 1983; Lebrun & Junter 1994):

$$\ln[C_0/(C_0 - 2C_r)] = 2At/VR_T \qquad (1)$$

where C_0 is the initial concentration in the donor compartment, C_r is the concentration in the receptor chamber at time t, V is the chamber volume, A is the exposed membrane area and R_T is the total mass-transfer resistance. Linear regression analysis of $\ln[C_0/(C_0-2C_r)]$ against time was used to estimate the total transport resistance. The total resistance includes the intrinsic membrane resistance (R_m) and the boundary layer resistance (R_b) on both sides of the membrane (Malone & Anderson 1977):

$$R_T = R_m + 2R_b = l/\varepsilon D_p + 2Rb \qquad (2)$$

where D_p is the apparent (effective) diffusion coefficient of the solute within the pores of the membrane, l is the membrane thickness and ε is the membrane porosity. R_b is a function of the stagnant-layer thickness, which in turn depends on the stirring conditions (Malone & Anderson 1977; Guillot et al 1985). If stirring is efficient the contribution of boundary resistance becomes negligible and D_p can be determined from R_T. A semi-empirical relationship between the diffusion coefficients and the molecular weights of the dextran standards was derived and used to estimate the molar mass of PS1A1.

Light scattering

A continuous-flow, dual-detector system (Dadey & Robbie, unpublished) was used to compare the molecular weight and self-association properties of PS1A1 (this laboratory) with those of a commercial dextran (Sigma) of broadly similar structure and size. This dextran had a weight-average molar mass of 73 kDa, as measured by the manufacturer by low-angle laser-light scattering with 0·1 M phosphate buffer (pH 7) as the mobile phase (personal communication). The mobile phase (Milli-Q water, 0·1 M NaCl solution or 0·1 M phosphate buffer pH 7) was clarified by vacuum filtration through a 0·1-μm polyvinylidene fluoride membrane filter (Millipore, Bedford, MA). Nitrogen-sparged mobile phase was pumped at 0·5 mL min^{-1} from a reservoir through a 0·1-μm in-line filter to the light-scattering and refractive-index detectors which were connected in series.

Samples of dextran or PS1A1 were added to investigate what effect sample concentration had on the molar mass of the compounds. Scattered light was detected with a mini-DAWN multi-angle laser-light-scattering photometer (Wyatt Technology, Santa Barbara, CA), operated with a semiconductor laser emitting vertically polarized light of wavelength 690 nm; the instrument was calibrated with toluene. Mass detection was performed with a Waters 410 differential refractometer (Millipore, Milford, MA), operated at 35°C. The refractive index increments (dn/dc) for dextran or PS1A1 were taken to be 0·147, 0·151 and 0·137 mL g^{-1} for solutions in water, 0·1 M sodium chloride and phosphate buffer, respectively (Huglin 1972). Signals from both detectors were digitized and acquired with Wyatt Astra software.

Elemental composition of PS1A1

Qualitative elemental analysis of PS1A1 in the dry, solid state was performed by energy dispersive X-ray spectroscopy using a Noran TN5402 EDX in conjunction with a JEOL JXA-35 scanning electron microscope. The acceleration voltage was 20 kV and the acquisition time 100 s. Background correction was not performed but the background count was known to be negligible relative to the signals generated.

Results

Diffusion technique

Figure 2 is a plot of $\ln[C_0/(C_0 - 2C_r)]$ against time for four dextrans of different molecular weight diffusing through 0·22-μm cellulose ester membranes. Linear regression analysis showed that the curves were linear ($P<0·05$) with correlation coefficients in the range 0·95–0·99. On the basis of equation 1 the slopes of these plots were $2A/VR_T$. Estimated values of the resistances of the membranes to mass transfer of dextrans of different molecular weights, and the effective diffusion coefficients of the four dextrans in the membrane pores, are shown in Table 1.

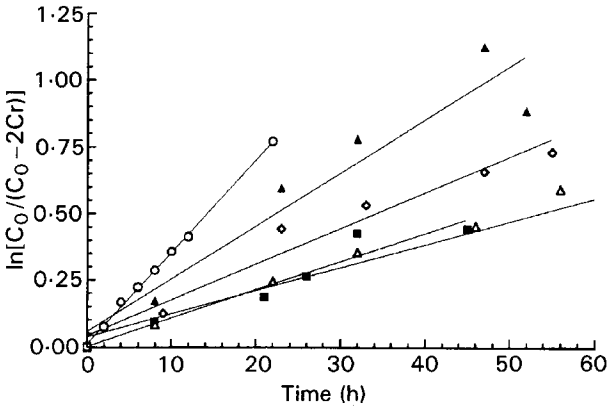

Figure 2. Linearized plots describing the diffusion through cellulose ester membranes (0·22-μm pore size) of PS1A1 (■) and of dextrans of different molecular weight: (○) $M_W = 9{\cdot}3$ kDa; (▲) $M_W = 19{\cdot}5$ kDa; (◇) $M_W = 39{\cdot}2$ kDa; and (△) $M_W = 73$ kDa. The graphs for PS1A1 and dextran 73 kDa almost overlap, indicating that the molar masses of these two polysaccharides are close to each other.

A linear relationship ($r = -0{\cdot}998$) was found between the effective diffusion coefficients of the dextran standards and their molecular weights raised to the power −0·66, according to the equation:

$$D_p = K_D M_W^{-b} = 2{\cdot}233 \times 10^{-6} \times M_W^{-0{\cdot}66} \quad (3)$$

The dextran concentration in the donor compartment was monitored to estimate the extent of membrane association, i.e. the amount of polysaccharide bound to the membrane by adsorption, trapped in the membrane pores, or passing through the membrane. The extent of association of the dextrans (Table 1) was calculated on the basis of conservation of mass.

The slope of a graph of $\ln[C_0/(C_0 - 2C_r)]$ against time (Figure 2, Table 1) was used to calculate the diffusion coefficient for transport of PS1A1 through the membrane pores; the value was $1{\cdot}541 \times 10^{-7}$ cm^2 s^{-1}. On the basis of equation 3 and the conservation of mass, the weight-average molecular weight of PS1A1 was 57·4 kDa, and the extent of membrane association was appreciably higher at 20% (Table 1).

Light scattering

The weight-average molecular weights of dextran in water, in 0·1 M NaCl and in 0·1 M phosphate buffer, as measured by multi-angle light-scattering, are given in Table 2. Statistical analysis by linear regression demonstrated there was no significant change ($P > 0{\cdot}05$) in the molecular weight of dextran with concentration in any of the solvents used. In addition, data evaluation by analysis of variance indicated that there was no significant difference ($P > 0{\cdot}05$) between the weight-average molecular weights of dextran in water, in 0·1 M NaCl solution and in 0·1 M phosphate buffer over the concentration range studied.

Figure 3 shows the weight-average molecular weight of PS1A1 in water and in 0·1 M NaCl, and of dextran in water over a concentration range 12·5–100 μg mL^{-1}. The molecular weight of PS1A1 was found to vary linearly ($r = 0{\cdot}990$; $P < 0{\cdot}01$) with concentration in water, but not in 0·1 M NaCl. In water, the weight-average molecular weight of PS1A1 varied from 564 to 1207 kDa over the concentration range 12·5–100 μg mL^{-1}, whereas in 0·1 M NaCl the molar mass of the same compound varied randomly between 60·1 and 68·1 kDa, with a mean of 65 kDa.

Elemental composition of PS1A1

The X-ray spectrum of PS1A1 (Figure 4) gave moderate proportionality peaks for carbon, oxygen and phosphorus, indicating the presence of these elements in the glucan. Other elements observed in the spectrum, e.g. Al, Na and Si, were present in

Table 1. Comparative data for dextrans and PS1A1 diffusing through the pores (0·22 μm) of cellulose ester membranes, including mass transfer resistance, diffusivity and extent of membrane association for the compounds.

Polysaccharide identity	Slope of plot of $\ln[C_0/(C_0 - 2C_r)]$ against time (s^{-1}) × 10^6	Overall diffusion resistance (s cm^{-1}) × 10^{-4}	Diffusion coefficient (cm^2 s^{-1}) × 10^7	Percentage (by mass) bound to the membrane
Dextran 9·3 kDa	9·614	3·646	5·143	1·8
Dextran 19·5 kDa	5·533	6·335	2·960	4·5
Dextran 39·2 kDa	3·761	9·320	2·012	5·8
Dextran 73·0 kDa	2·421	14·480	1·295	7·6
PS1A1	2·960	12·170	1·541	20·0

Table 2. Weight-average molecular weight of dextran in various solvents as determined by static light-scattering.

Addition number	Concentration (μg mL^{-1})	Molecular weight of dextran (kDa)		
		Milli-Q water dn/dc = 0·147 mL g^{-1}	0·1 M NaCl dn/dc = 0·151 mL g^{-1}	Phosphate buffer dn/dc = 0·137 mL g^{-1}
1	150	76·3 ± 3·0	73·9 ± 5·0	77·9 ± 7·0
2	275	74·8 ± 2·0	71·5 ± 5·0	73·7 ± 6·0
3	400	73·9 ± 2·0	70·5 ± 5·0	73·4 ± 6·0

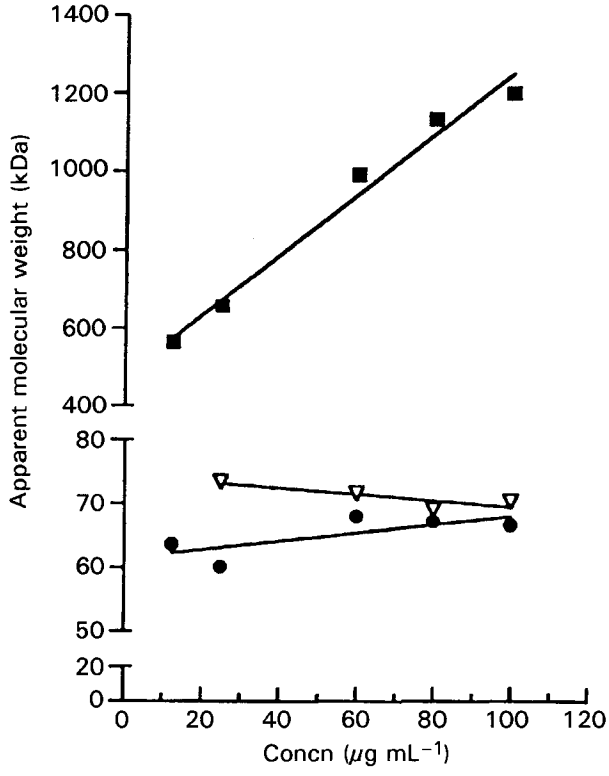

Figure 3. Concentration-dependence of the weight-average molecular weights of PS1A1 in water (■) and in 0·1 M sodium chloride solution (●), and of dextran (73 kDa) in water (▽), as measured by multi-angle laser-light scattering.

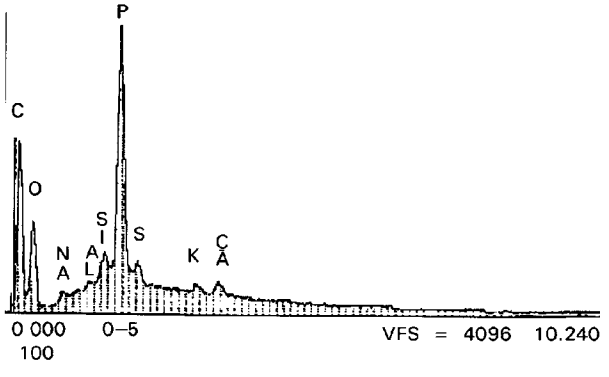

Figure 4. Qualitative X-ray spectrum of PS1A1 showing the elemental composition of the glucan. The peaks for carbon, oxygen and phosphorus fall in the moderate proportionality range (peak area 10 000–30 000 counts).

trace amounts and were contaminants from the extraction process.

Discussion

The various techniques used for determination of molecular weight can be classified into absolute and relative processes. In absolute methods (such as light scattering, membrane osmometry and mass spectrometry), the molecular weight of the polymer is calculated directly from measured quantities, which does not require a knowledge of physical properties or chemical structure. Relative methods measure properties such as viscosity and diffusion coefficients that are dependent on this characteristic of the macromolecule (Elias 1977). These methods require calibration by use of appropriate standards whose molar masses have been measured using an absolute process.

Initial studies dealing with the relationship between diffusion coefficients and molecular weights were performed by Euler (1897). Using simple, low molecular-weight sugars and salts Euler observed that diffusion coefficients were inversely proportional to the square root of the molecular weight. As reviewed by Duclaux (1936), Tholvert demonstrated that Euler's theory was not applicable to all substances, suggesting that for unrelated molecules a change in molar mass might fail to result in a proportionate change in molecular dimensions. Thus, a relationship between molar mass and diffusivity might be considered only for identical structurally related compounds. In our diffusion experiment, commercial dextrans were used to construct calibration curves from which the molecular weight of PS1A1 seems to be underestimated by this technique by approximately 12%. This might be because the diffusion coefficient of a polymer through a membrane pore is dependent on molecular dimensions, configuration (shape) and deformability, as well as on the interaction between the polymer molecules and the solvent. Although PS1A1 and dextrans are both similar glucose homopolymers with α-(1 → 6)-linked main chains, our results suggested that the configuration of

Table 3. Comparison of the molecular weights of PS1A1 measured by different techniques.

Technique	Solvent and mobile phase	Molecular weight (kDa)
Size-exclusion chromatography (Wang et al 1995)	Distilled water	65–87
Diffusion through porous membranes	Distilled water	57·4
Static light-scattering	1. Distilled water	564–1207
	2. 0·1 M NaCl solution	60–68 (mean 65)

PS1A1 molecules differed slightly from that of dextrans, especially in view of the known minor differences between the branch linkages, branch lengths and branching frequency of the two entities. In PS1A1 molecules, the side-chains occur every five to six residues along the main chain (Wang et al 1995), but the branches are only one glucose unit long. On the other hand, in dextrans, some of the branches (<15%) are more than two monomers long (Larm et al 1971). The shorter side-chains of PS1A1 might explain why diffusion occurred more rapidly than for dextran, thereby leading to an underestimation of the molecular weight of PS1A1. Dextran molecules, with short side-chains, are also known to be flexible (Bohrer et al 1984). It is therefore probable that PS1A1 molecules would be even more flexible and, therefore, better able to pass through the filter pores. This observation is also consistent with the observed difference in membrane association, Table 1.

Differences between the solution behaviour of PS1A1 and dextran were also noted in the light-scattering experiments. PS1A1 was observed to undergo concentration-dependent multimerization in water, whereas no significant self-association was observed for dextran under the same conditions. This suggests that solute–solute and solute–solvent intermolecular interactions in water are different for the two polysaccharides. On the basis of these observations it was concluded that although the diffusion technique provided an approximation of the molar mass of the BCG-derived polysaccharide absolute techniques were preferable for evaluation of the molecular weight of PS1A1. Static light-scattering was used to determine the molar mass of this glucan but the estimation was complicated by the association of PS1A1 in water. Although size-exclusion chromatographic studies had shown PS1A1 to be an α-glucan of molecular weight approximately 65 to 87 kDa (Wang et al 1995), it was evident that aggregation was suppressed by the presence of electrolyte. Measurements by light scattering employing a sodium chloride solution as solvent and mobile phase resulted in an estimated molecular weight of the PS1A1 monomer of 65 kDa. Table 3 summarizes the molecular weights measured for this glucan by different techniques.

The effect of salt on the average molar mass of PS1A1 implies that the intermolecular interactions between the polysaccharide molecules are weakly electrostatic, rather than hydrophobic, in nature. Elemental analysis of PS1A1 showed that, in addition to the carbon, hydrogen and oxygen expected from its polysaccharidic structure, PS1A1 also contained phosphorus. The phosphorus was not detected by NMR analysis performed by Wang et al (1995), although the NMR conditions were not ideal for detecting this element. The amount of phosphorus present in the structure is currently not certain, because the X-ray technique is qualitative and peak height or area cannot be used to assess the relative proportion of the elements present. This is because the peak size depends not only on the abundance of the element, but also on atomic size and the ease with which electrons can be knocked off the molecules by the incident X-ray beam. We believe, therefore, that the structure of glucan PS1A1 might include previously unobserved ionized phosphate groups which could be involved in the electrostatic intermolecular interactions and are responsible for the aggregation in water.

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