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Physicochemical studies of pectin/poly-L-lysine gelation

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Abstract—The effect of poly-L-lysine concentration and degree of polymerisation on the gelation of pectins differing in charge density and distribution was examined, through the determination of gel stiffness, swelling behaviour and the binding of poly-L-lysine to the gel network. Poly-L-lysine acts as a crosslinker of concentrated pectin solutions, with its effectiveness showing dependencies on pH and charge distribution on the pectin. Neutralisation of the anionic charge on the pectin with the polycationic peptide leads to gel opacity and eventually network collapse.

Keywords: Pectin; Poly-L-lysine; Gelation

1. Introduction

Pectic polysaccharides are components of the primary cell wall and middle lamella of dicotyledonous plants.[1] They are structurally complex and heterogeneous polyelectrolytes, consisting of linear regions of (1→4)-α-D-galacturonosyl units and their methyl esters, interrupted in places by (1→2)-α-L-rhamnopyranosyl units. A fraction of these rhamnopyranosyl residues are branch points for neutral sugar side chains of (1→5)-α-L-arabinofuranosyl or (1→4)-β-D-galactopyranosyl residues.[2,3] On the basis of their methoxyl content pectins can be described as high methoxyl (HM), with a degree of esterification (DE) > 50%, and low methoxyl (LM) (DE < 50%). The distribution of charged uronic acid residues along the pectin backbone also has an important influence on physicochemical behaviour. The charge may be randomly distributed, or may occur in blocks of contiguous uronic acid residues.

Pectins extracted from cell wall materials under mild conditions are very large macromolecules and form entangled solutions at relatively low pectin concentrations (~0.2%). Gels can form from these entangled solutions through interactions between pectin chains at junction zones. A HM pectin can gel at low pH (~3) in the presence of a large concentration of co-solute (for example sucrose), while a LM pectin forms gels at higher pH (5–7) in the presence of Ca^{2+} ions. This classification is not clear cut. It was recently shown that a HM cell wall pectin, containing blocks of unesterified residues, can also gel on addition of Ca^{2+} ions.[4] In this case it was proposed that the blocks of unesterified residues played a role in network crosslinking, while more isolated residues made a contribution to network swelling through a polyelectrolyte effect.

The affinity of pectin for the cations found in the apoplast increases in the order $K^+ < Mg^{2+} < Ca^{2+}$.[5,6] The affinity for Ca^{2+}, and other cations increases with increasing charge density along the polymer backbone and increasing 'blockiness'.[7,8] Ca^{2+} affinity for the pectin chain is higher in gels than in solutions[7] and is related to the higher affinity of the crosslinking junction zone for Ca^{2+}, than the isolated chain in solution. Compared to the calcium-mediated gelation of pectic polysaccharides, relatively little work has been published about the interactions of pectin and organic cations. These interactions are relevant to the understanding of the pectin network of the plant cell wall, as well as for industrial applications of these networks. Dilute solution

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studies[9–11] have shown that the interaction of basic peptides with polygalacturonate can induce a conformational change. It has also been shown[12] that addition of basic peptides (including poly-L-lysine, poly-L-arginine and synthetic peptide fragments of the cell wall protein extensin) to a cell wall pectin resulted in gel formation. Peptide addition had an effect on both network crosslinking and swelling. At high levels of addition network collapse was observed, as the charge on the polyanion (pectin) was neutralised by the charge on the polycation (peptide).

In this paper we investigate the interaction between pectic polysaccharides and poly-L-lysines of different chain lengths, and the effect of this interaction on network crosslinking and swelling.

2. Experimental

2.1. Source of materials

Citrus pectins were obtained from CP Kelco. Poly-L-lysines were obtained from Sigma, the reported average molecular weights were 500–2000 Da (determined from capillary electrophoresis), 2900 and 9800 Da (determined from viscosity) that indicate average degrees of polymerisation (DP) of 4, 14 and 47, respectively.

2.2. Pectin analysis

The fragmentation of the pectins, and the subsequent analysis of the oligomeric products by high pressure anion exchange chromatography (HPAEC) was carried out as described.[13]

2.3. Viscometry

Measurements of specific viscosity as a function of concentration of pectin (0.01–0.1% w/w) in 50 mM acetate buffer (pH 5.6) were carried out using an Ubbelohde viscometer at 20 °C. The efflux time for buffer was 115 s and no shear rate corrections were made. The intrinsic viscosity [η] was calculated from the extrapolation of reduced viscosity to zero concentration.

2.4. Preparation of gels and measurement of gel stiffness

Gel formation was carried out at 4 °C. Cylindrical gels (8 mm diameter) were formed by weighing ~300 mg of 3% w/w pectin solution in 50 mM acetate buffer (pH 5.6) into a straight-sided 2 mL polyethylene centrifuge tube. The pectin solution was acidified to pH ~ 2 by addition of HCl and after 30 min poly-L-lysine was added. After 8 h, the pH of the mixture was adjusted to ~5.6 by addition of 5 M NaOAc. The gels were examined after a further 16 h at 4 °C. The stiffness of the gels at 20 °C was determined as the shear modulus (G) at 200 Hz calculated from the measured velocity of a shear-wave passing through the gel using a Rank pulse shearometer.[14]

2.5. Poly-L-lysine binding behaviour

For studying the binding of poly-L-lysine to the poly-L-lysine/pectin gel network, 300 mg gels were formed and immersed for 24 h in 1 mL of distilled water at room temperature (20 °C). The amount of free poly-L-lysine in the leachate was determined by size exclusion chromatography using a BioSep-SEC-S 2000 column operating at a flow rate 1 mL/min with phosphate buffer as the eluant, and a UV detector (λ 210 nm). The column was calibrated using poly-L-lysine standards.

2.6. Swelling measurements

The swelling behaviour of gels and pectin solutions (3% w/w) was determined as a function of osmotic stress.[15] Solutions of various concentration of PEG 20,000 were prepared to provide osmotic pressures from 50 to 5×10^6 Pa.[16] Poly-L-lysine-pectin gels were prepared in dialysis tubing, immersed in 10 mL PEG solution and equilibrated for 24 h. The swollen gels were then weighed and their stiffness measured. The swelling behaviour of pectin solutions was carried out in a similar way.

3. Results and discussion

3.1. Preliminary experiments

In earlier experiments, a tomato pectin, with a uronic acid content of 83% and a degree of methyl esterification of 65%, was shown to gel with basic peptides.[12] We have now examined the ability of pectins from other sources to form these gels. The characteristics of the pectins chosen for the study are outlined in Table 1. The uronic acid content ranged from ~87% to ~94% w/w with degrees of esterification ranging from ~20% to 60%. The neutral sugars present were predominantly galactose, with smaller amounts of arabinose and rhamnose. Pectins 0001-8-B (DE 57.7%, enzyme de-esterified) did not gel on addition of poly-L-lysine DPs 4 and 14, and formed very weak viscoelastic gels with poly-L-lysine DP 47. Of the pectins with a DE of ~36%, 98246-5-E (chemically de-esterified) formed a weak viscoelastic gel with poly-L-lysine DP 47 while pectin 0001-8-F (enzyme de-esterified) formed elastic gels with poly-L-lysine (DP 4, 14 and 47). Pectin 98246-5-G DE ~20% (chemically de-esterified) was also examined. Opaque gels were formed, which syneresed, indicating polymer aggregation and precipitation. These results suggest that the poly-L-lysine/pectin interaction is sensitive to both the extent and the pattern of distribution of esterified units.

Table 1. Chemical composition of pectins

Sample	Type of de-esterification	Anhydrosugar (μg) per mg dry weight							%DE	%GalA
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc		
98246-5-E	Chemical	6.9	0.0	2.1	1.3	0.0	37.0	2.7	35.6	92.4
98246-5-G	Chemical	6.0	0.0	0.3	1.5	0.0	21.0	1.7	20.6	94.3
0001-8-B	Enzyme	10.7	0.0	27.0	1.9	2.2	64.4	7.4	57.7	87.3
0001-8-F	Enzyme	7.1	0.7	15.6	1.7	0.2	49.0	3.6	36.7	89.1

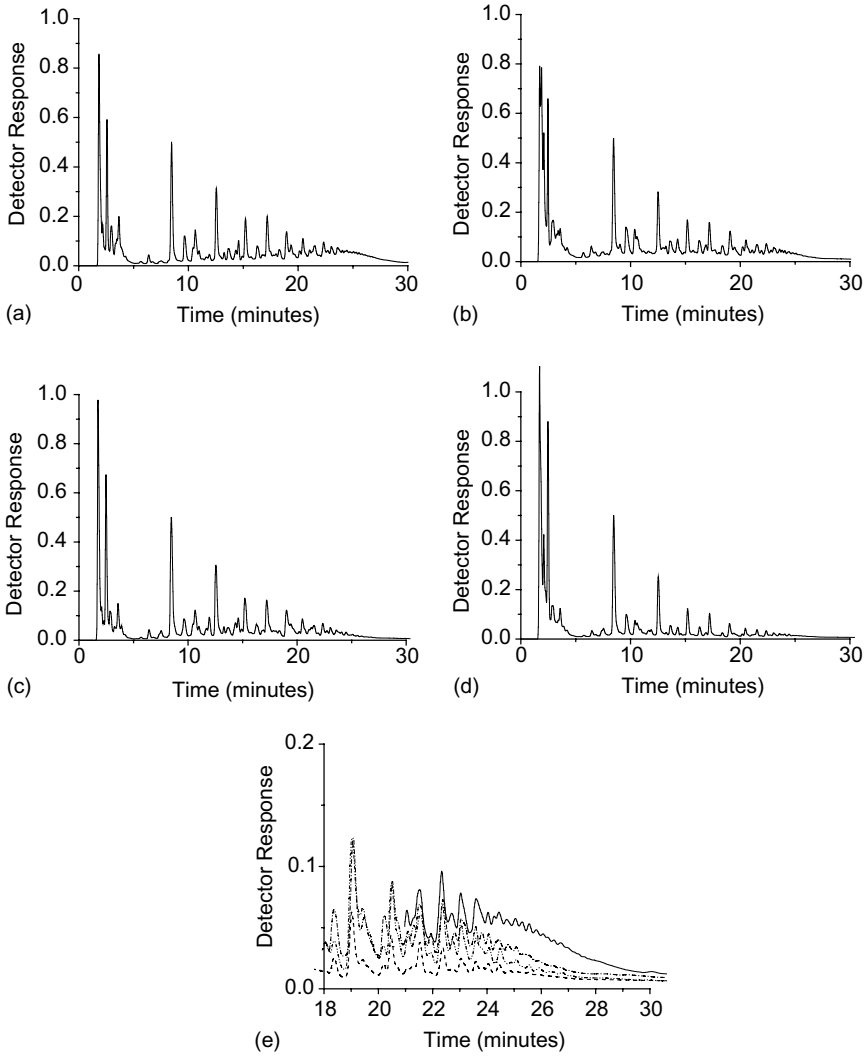

Figure 1. HPAEC at pH 6 of fragmented pectins 98246-5-G (a); 0001-8-F (b); 98246-5-E (c); 001-8-B (d) and a comparison of fragmentation profiles (e) for 98246-5-G (—); 0001-8-F (-·-); 98246-5-E (-···-); 001-8-B (- - -).

The pattern of distribution of the uronic acid in the pectin was examined using a chemical fragmentation procedure.[13] The methyl esterified uronic acid derivatives are degraded. Partially esterified pectins are specifically cleaved to generate a series of oligogalacturonic acid residues bearing an arabitol residue as aglycone. Chromatographic analyses of the fragments produced are shown in Figure 1, for samples 98246-5-G (a); 0001-8-F (b); 98246-5-E (c); 001-8-B (d). Each chromatogram shows a series of major peaks, $(GalA)_n$–1,5-di-D-Araol, with smaller amounts of by products, the structure of which are discussed in Needs et al.[13] The structure of the major components was confirmed by negative-ion ESI-MS. The major observed difference in the profiles obtained is in the amount of higher molecular weight oligomers obtained with degrees of polymerisation >8, at retention times in the range 18–30 min. In order to permit comparison, this portion of the chromatograph was enlarged after normalising the traces to the peak area of $(GalA)_2$–1,5-di-D-Araol (retention time ~8 min).

The pattern of distribution of uronic acid in the pectin molecule was not markedly associated with the method of de-esterification. The pectin (98246-5-G), which formed an opaque, syneresing gel had the largest fraction of higher molecular weight oligomeric fragments. The pectin (0001-8-B), which only formed a very weak gel with poly-L-lysine DP 47, had the smallest fraction of higher molecular weight oligomeric fragments. Gelation behaviour was associated with the presence of this fraction.

3.2. Gel formation and properties

The behaviour of pectin 0001-8-F was examined in more detail. The measured intrinsic viscosity, $[\eta]$, at pH 5.6 was 346 mL/g. According to reference data[17] the product of intrinsic viscosity and coil overlap concentration, C^*, is constant ($C^*[\eta] = 0.77$), giving a value of ~0.2% for C^*. Poly-L-lysine/pectin gels were prepared at pectin concentrations >0.5% w/w—above the coil overlap concentration. The gels were elastic solids, which recovered from small static deformations. At peptide–pectin charge ratios of <0.56 the gels were clear. With increasing concentration of peptide, some opacity developed, showing a tendency for polymer aggregation and precipitation on charge neutralisation.

The influence of pH on gel stiffness was examined (Fig. 2) for a 3% w/w pectin solution, crosslinked with 0.46 mM poly-L-lysine DP 47 (peptide–pectin charge ratio 0.32). Over the pH range studied, gel stiffness increased with increasing pH from 605 N/m^2 at pH 4.1 to 2990 N/m^2 at pH 6.1. With increasing degree of dissociation of the carboxyl groups of the pectin (the pK_a of the 'monomer', α-D-galacturonic acid is 3.4[18]), there is increased opportunity for poly-lysine binding. In the pH range 5.25–5.75 there was a weak dependence of gel stiffness on pH, and for this reason other experiments were carried out at pH 5.5.

The effect of poly-L-lysine concentration and DP on gel formation for a 3% w/w pectin solution was examined (Fig. 3). For all the peptides, gel stiffness increased with increasing concentration of poly-L-lysine, went through a maximum, and then fell somewhat. The maximum modulus was observed at peptide–pectin charge ratios of 0.21, 0.32 and 0.56 for poly-L-lysine of DP 4, 17 and 47, respectively. At higher concentrations, the increasing neutralisation of charge on the network by the polycation leads to network opacity and collapse. On a molar basis the most effective crosslinking agent was poly-L-lysine DP 47, followed by poly-L-lysine DP 14 and 4. The peptides can be more effective crosslinkers than Ca^{2+}. Typically 1–2 mM Ca^{2+} is necessary to induce gelation,[19] while for poly-L-lysine DP 47 ~ 0.03 mM is sufficient. These results demonstrate that the effectiveness of the peptide as a crosslinker is chain length dependent, and suggests that there is a sufficient length of unsubstituted uronic acid residues on the pectin chains in the crosslink to form an interaction with poly-L-lysine DP 47. The dependence of shear modulus, G, on polymer concentration from 0.5% to 5% w/w was studied for gels consisting of poly-L-lysine DP 47, with a constant peptide–pectin charge ratio of 0.32. A linear dependence between stiffness and polymer concentration was observed in this range (Fig. 4).

In order to examine the role of peptide–pectin interactions on gel formation, it is useful to estimate the number of crosslinks in these systems. From the theory of rubber elasticity for crosslinked synthetic polymer networks[20,21] the dependence of shear modulus on molecular weight between crosslinks, M_c, is given by

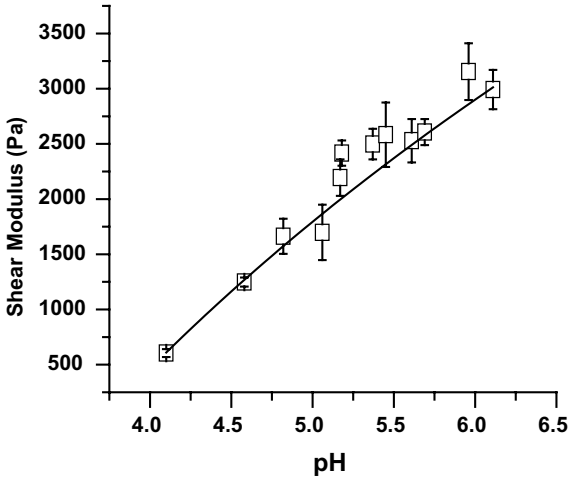

Figure 2. Dependence of the shear modulus G' on the pH of gels formed from 3% w/w pectin solution in 50 mM acetate buffer and 0.46 mM poly-L-lysine DP 47.

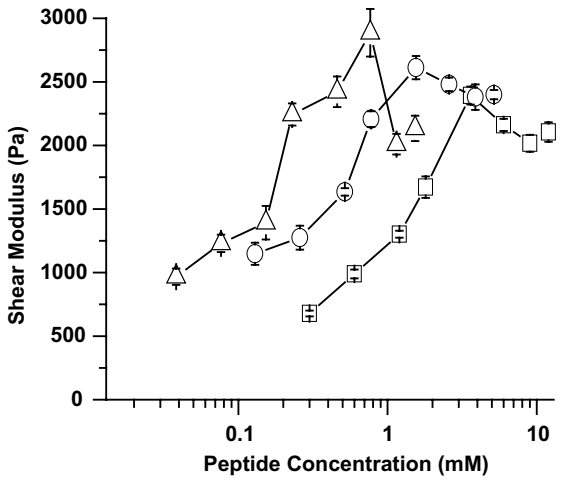

Figure 3. Dependence of shear modulus G' on poly-L-lysine concentration (DP 4 (□); DP 14 (○); DP 47 (△)) in gels formed from a 3% w/w pectin solution in 50 mM acetate buffer at pH 5.6.

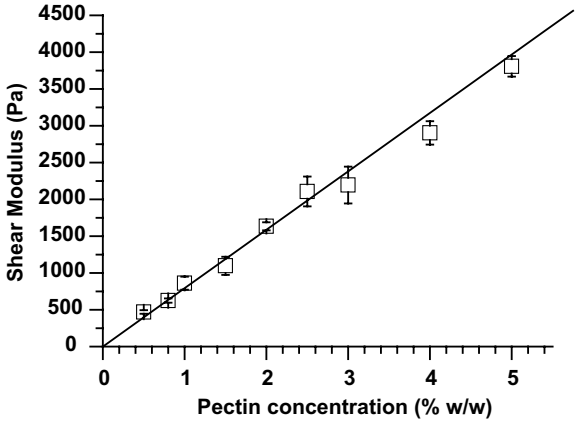

Figure 4. Dependence of shear modulus on pectin concentration for gels crosslinked with at a constant peptide–pectin charge ratio of 0.32.

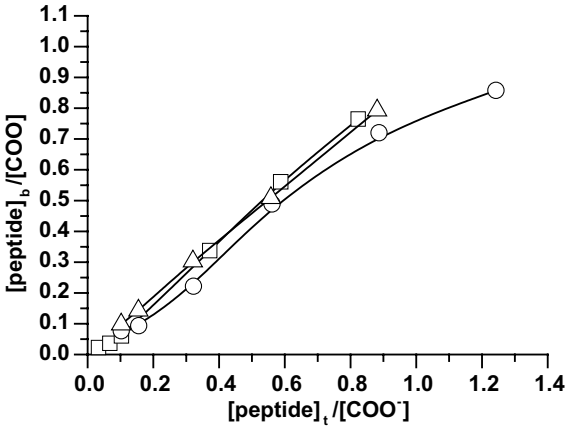

Figure 5. Poly-L-lysine binding behaviour (DP 4 (□); DP 14 (○); DP 47 (△)) to a 3% w/w pectin gel in 50 mM acetate buffer (pH 5.6).

$$G' = \frac{cRT}{M_c}, \quad (1)$$

where c is the polymer concentration, and R and T have their usual meanings. According to this theory the mechanism of energy storage is entropic, arising as a result of a constraint on the number of accessible configurations on the polymer as the network is stretched. For poly-L-lysine pectin gels with a 3% w/w pectin concentration at pH 5.5, M_c varies from $\sim 1.1 \times 10^5$ for a sample with 0.3 mM poly-L-lysine DP 4 (peptide–pectin charge ratio 0.017) to $\sim 2.5 \times 10^4$ for a sample with 0.77 mM poly-L-lysine DP 47 (peptide–pectin charge ration 0.56) and depends both on the type and concentration of poly-L-lysine. These values correspond to only a few crosslinks per macromolecule.

3.3. Poly-L-lysine binding behaviour in poly-L-lysine/pectin gels

The poly-L-lysine binding behaviour in poly-L-lysine/pectin gels was examined by determination of free poly-L-lysine. The experimental data are presented as binding isotherms $[peptide]_b/[COO^-]$ versus $[peptide]_t/[COO^-]$, where $[peptide]_b$ is the molar concentration of bound poly-L-lysine monomer, $[peptide]_t$ is the total molar concentration of poly-L-lysine monomer, and $[COO^-]$ the molar concentration of uronic acid. The binding behaviour was examined both as a function of peptide concentration and peptide DP (Fig. 6).

The binding behaviour shows little tendency to reach a plateau at $[peptide]_t/[COO^-]$ ratios <1.2. The binding behaviour was analysed using the Hill equation:[22]

$$\log L = -\frac{1}{\alpha_H} \log\left(\frac{n_H}{v} - 1\right) + \log K_H, \quad (2)$$

where v is the amount of poly-L-lysine bound by a pectin segment and L the concentration of free poly-L-lysine, and n_H is the number of sites per segment able to bind cations. It was convenient for the comparison of binding behaviour, to choose as the pectin segment, a unit containing 4, 14 and 47 carboxyl groups binding just one poly-L-lysine cation with a DP 4, 14 and 47, respectively (a 1:1 complex). K_H is the apparent dissociation constant for the interaction between the peptide and the pectin segment ($1/K_H$ is the stability constant K_B), and α_H the Hill constant, which is an index of the cooperativity. When $\alpha_H > 1$, the binding is cooperative and anticooperative when $\alpha_H < 1$. The Hill plots for poly-lysines are presented in Figure 5, with values for α_H, n_H and K_B being given in Table 2. The values for α_H indicate a high degree of cooperativity, and are larger than the value obtained for Ca^{2+} binding.[8] The values of K_B are between 775 and 8700, and are comparable to those observed for the binding of Ca^{2+} to pectin in solution or gel.[7] The values of n_H obtained (0.91–0.98) are close to those expected for the 1:1 binding of poly-lysine to an appropriately sized pectin segment. Comparison of the binding and elastic behaviour of the gels indicates that relatively few of these complexes function as crosslinks.

Table 2. Binding parameters for the interaction between poly-L-lysine and pectin

	α_H	n_H	K_B	R^2
Poly-L-lysine DP 4	4.61	0.91	775	0.979
Poly-L-lysine DP 14	3.12	0.94	1262	0.993
Poly-L-lysine DP 47	4.50	0.98	8700	0.834

3.4. Swelling behaviour

A characteristic of polyelectrolyte gels is their swelling behaviour in aqueous solution. This swelling is largely governed by an imbalance in distribution of mobile counterions between the gel and its surrounding solution

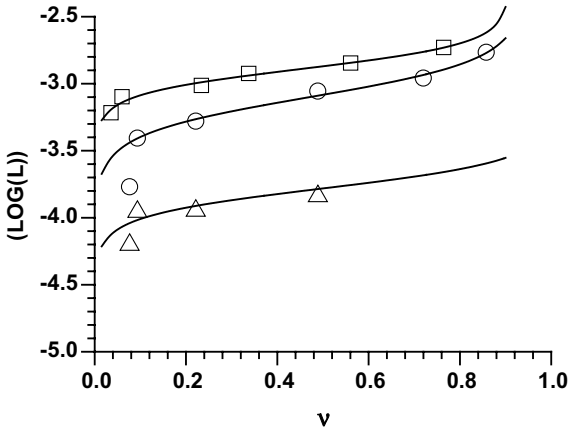

Figure 6. Hill plot of binding behaviour of poly-L-lysine (DP 4 (□); DP 14 (○); DP 47 (△)) to a 3% w/w pectin gel in 50 mM acetate buffer (pH 5.6).

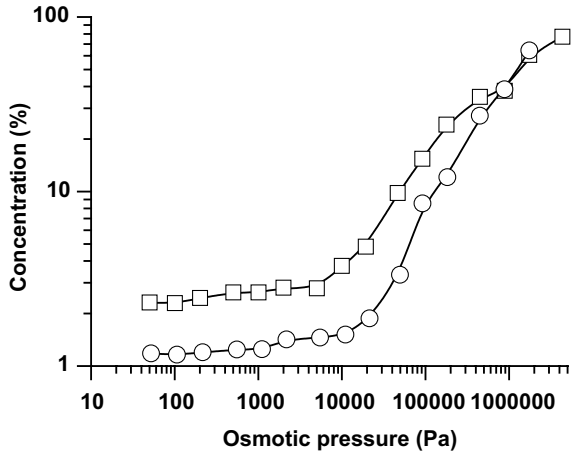

Figure 7. Concentration dependence of osmotic pressure for a pectin solution (○) and a crosslinked poly-L-lysine/pectin gel (□).

due to a Donnan effect.[21–23] The osmotic pressure in a polyelectrolyte network is given by

$$\pi \cong \left(\frac{1}{\xi^3} + \frac{c^2}{A(c + 4Ac_s)}\right)RT, \qquad (3)$$

where ξ is the correlation blob size, c the monomer concentration, c_s the salt concentration, and A the number of monomers between uncondensed charges.[21] This relationship applies to 1:1 electrolytes. The first term is the polymer contribution to swelling (arising from the configurational entropy of chains) and the second term is due to translational entropy of free ions. For a 1:1 electrolyte $S^* = c_s$, where S^* is ionic strength. The polymer concentration c can be represented as c_0/q, where q is the extent of equilibrium swelling and c_0 the initial monomer concentration. At sufficiently low polymer concentrations (the concentration of anhydrogalacturonic acid residues in a 3% w/w solution is 0.09 M) the ionic term dominates over the polymer term. In this case

$$\pi \cong \frac{RTc_0^2}{q^2A\left(\frac{c_0}{q} + 4AS^*\right)}. \qquad (4)$$

The effect of osmotic stress on the concentration of pectin solutions and poly-lysine/pectin gels was examined at pH 5.6 in 50 mM acetate buffer (Fig. 7). At a fixed osmotic stress, pectin in solution is less concentrated than pectin in the gel network. The solution behaviour of pectin is reasonably described by relationships of the form of 4 with a fitted value for A of 2.27 (Fig. 8a). The value of A obtained for the average spacing between charges indicates that only a fraction of the charge on the pectin contributes to the polyelectrolyte swelling effect. It is noticeable that even pectins, which are enzyme de-esterified still have isolated uronic acid residues, or very short lengths of contiguous

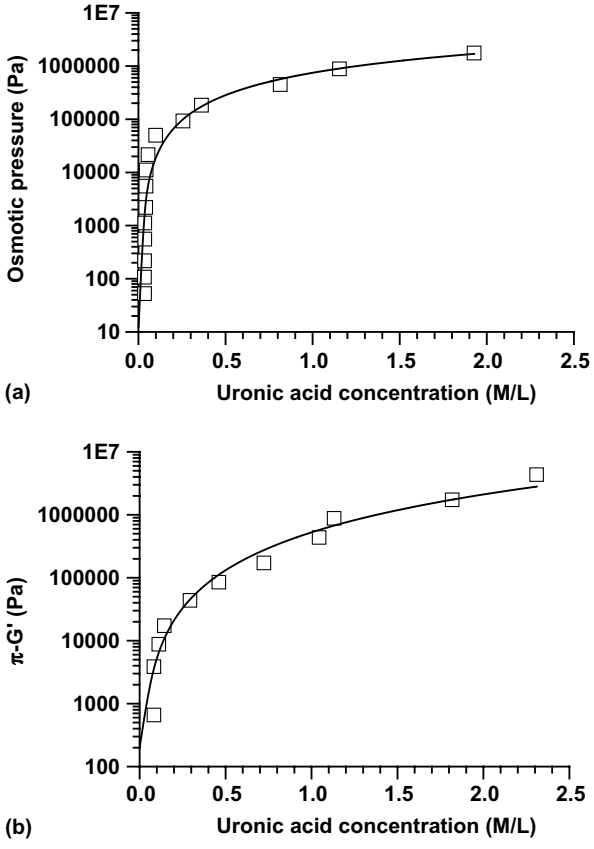

Figure 8. (a) Comparison of observed (□) and predicted (—) behaviour for the concentration dependence of osmotic pressure for a pectin solution. (b) Comparison of observed (□) and predicted (—) behaviour for the concentration dependence of osmotic pressure of a poly-L-lysine/pectin gel.

unesterified uronic acid residues (dimer, trimer), which can potentially play a role in network swelling (Fig. 1). It is well known that for highly charged polyelectrolytes the phenomenon of counterion condensation can lead to

similar effects.[24,25] To estimate whether counterion condensation should be observed a dimensionless parameter ς is calculated from

$$\varsigma = \frac{e^2}{\varepsilon\varepsilon_0 4\pi kTb},$$

where ε is the dielectric constant of the medium, ε_0 the dielectric constant, e the elementary charge, k Boltzmann's constant, and b the spacing between charges. For many polyelectrolytes, counterion condensation is observed above a value $\varsigma = 1$. For water at 25 °C, $\varsigma = 0.71/b$ if b is expressed in nm. With a charge spacing along the polygalacturonic acid chain of 0.435 nm,[5] the calculated value of $\varsigma = 1.63$ indicates that counterion condensation should occur until the condensation reduces the effective charge spacing to the value $\varsigma = 1$. The predicted spacing between effective charges of 1.63 is in satisfactory agreement to that found from swelling experiments 2.27. The observed swelling behaviour of the pectin in solution is consistent with the view that the presence of blocks of charge on the pectin leads to counterion condensation and an increase in the effective separation between charges.

In the case of poly-L-lysine/pectin gels a number of physicochemical factors might be expected to influence the observed behaviour. Firstly the ionic strength is higher, 0.9 M compared to 0.05 M in solution. This is related to the method of preparation of the gel with the initial acidification followed by neutralisation. It also includes a contribution from the polycation poly-L-lysine. In the limit of high salt concentration Eq. 4 reduces to

$$\pi \equiv \frac{c^2RT}{4A^2c_s} = \frac{c_0^2RT}{4q^2AS^*}. \quad (6)$$

The total free energy for the gel consists of two parts: an osmotic part F_{os} (determined by the osmotic pressure inside the gel) acting to oppose the imposed osmotic stress, and an elastic part F_{el} that similarly opposes the applied osmotic stress. Equilibrium swelling behaviour is determined when the total free energy is minimised with respect to volume. The derivative of the elastic term is the modulus ($G \cong \partial F_{el}/\partial V$). Therefore at equilibrium

$$\pi = \frac{c_0^2RT}{4q^2A^2S^*} + G. \quad (7)$$

Taking into account the contribution of gel elasticity in opposing the applied osmotic stress, and assuming that there is no net change in crosslinking, it is possible to calculate the average spacing between charges, which contribute to the swelling of the poly-L-lysine/pectin gel (Fig. 8b). The value of A obtained of 4.29 reflects involvement of some of the charged uronic acid residues in network crosslinking and poly-L-lysine/pectin interactions reducing network charge. This latter effect was investigated in more detail through examination of the effect of progressive addition of polycationic peptide on the observed swelling behaviour at a fixed osmotic stress of 100 kPa (Fig. 9). Increasing concentration of peptide of DP 4 and 47 progressively reduced swelling. On a molar basis poly-L-lysine DP 47, was more effective in suppressing swelling, although the range of peptide–pectin charge ratios was broadly similar for both peptides, 0.05 to 0.8 for DP 47 and 0.07 to 1.2 for DP 4. At a fixed charge ratio, the shorter peptide was more effective in reducing swelling. An explanation for this effect, is that more of the monomeric units of the DP 47 are not able to bind to the charge on the pectin chain because of the charge distribution on the backbone.

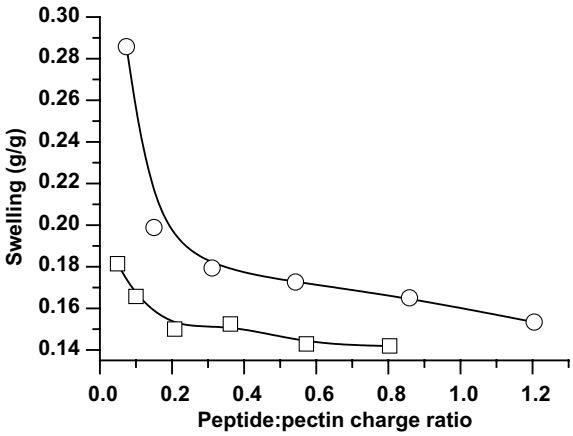

Figure 9. Dependence of the swelling of a poly-L-lysine/pectin gel on peptide–pectin charge ratio (DP 4 (□); DP 47 (○)) at a fixed osmotic stress of 100 kPa.

These data illustrate that the addition of polycations to an anionic pectin gel can have a marked effect on swelling behaviour, and is dependent on charge ratio of the polyions, chain length of polycation and charge distribution on the polycation.

4. Conclusions

Poly-L-lysine can act as an effective crosslinker of pectin networks at pH's close to neutrality. Clear elastic gels were formed from a pectin with a degree of methyl esterification of 36% in which the charge distribution occurred in blocks. Gel stiffness increased with increasing concentration of crosslinker and increased to a maximum at peptide–pectin charge ratios in the range 0.21–0.56. Further addition of polycationic peptide leads to a loss of gel clarity and eventually network collapse. The affinity of the peptide chain for the pectin increases with increasing chain length of peptide with the stability constant for the interaction reaching 8700 for a peptide of DP 47. In addition to its role as a crosslinker, the presence of the peptide reduces the

magnitude of the Donnan effect, which drives network swelling. In the complex environment of the apoplast the overall balance of ionic effects, involving both inorganic and organic cations, can modulate the properties of the pectin network through their effect on network cross-linking and swelling.

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