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Pectin–chitosan interactions and gel formation

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Abstract—The effect of chitosan concentration on the gelation of pectins differing in charge density and distribution was examined, through the determination of gel stiffness and the binding of chitosan to the gel network. Chitosan acts as a crosslinker of concentrated pectin solutions, with its effectiveness showing a dependency on charge on the pectin. The networks produced are clear even under conditions of charge neutralisation.

Keywords: Pectin; Chitosan; Gelation

1. Introduction

Biopolymers, including polysaccharides, proteins and nucleic acids, are often polylectrolytes. They have important technological applications as thickeners, gelling agents and coatings. Weakly charged polyelectrolytes are typically water soluble at low ionic strength in solutions containing monovalent counterions, because of a net repulsion between charged monomers. Multivalent counterions modify the stability of semi-concentrated solutions,[1–3] through the formation of strong attractive interactions, which can lead to network formation and gelation. The state of the polyelectrolyte system (gel, collapsed system, phase separated system) greatly depends on polyelectrolyte charge density, counterion concentration and type, and any specific interaction between the polyelectrolyte and the counterion.[4] The charge density is characterised by a dimensionless charge density parameter,[5,6] ξ,

$$\xi = \frac{e^2}{\varepsilon\varepsilon_0 4\pi kTb}, \quad (1)$$

where ε is the dielectric constant of the medium, ε_0 the dielectric permittivity of free space, e the elementary charge, k Boltzmann's constant, T is the absolute temperature and b is the average distance between neighbouring charge groups on the polymer backbone. At high charge densities when $\xi < 1$, counterions 'condense' on the polymer backbone.

Although most polyelectrolyte theory is developed and applied to synthetic polyelectrolytes,[7–10] the physical insights gained are equally relevant to the study of biopolymers. Pectins are anionic polysaccharides, extracted from plant cell walls.[11] They are composed of a $(1\rightarrow4)$-α-D-galacturonic acid backbone interrupted with $(1\rightarrow2)$-L-rhamnose residues. Neutral sugars, typically D-galactose and L-arabinose, occur as short side chains attached to C-4 of the rhamnosyl residue.[12] The uronic acid residues of native pectins may be partially methyl esterified. The distribution of charged uronic acid residues along the pectin backbone can also be modified using different methods of de-esterification. Chemical de-esterification produces a random distribution of charged residues. Enzyme de-esterification has the potential to produce a random distribution of charge if the enzyme/substrate complex dissociates immediately after hydrolysis of the ester, or it may produce a more blockwise distribution of charge if the enzyme progresses along the pectin chain progressively hydrolysing the methyl ester from contiguous residues. Pectins can therefore have both different average charge densities and charge distributions.

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Polysaccharide gels form through the physical association of polymer chains at junction zones to form an interconnected network. Low methoxyl pectins (degree of esterification, DE, <50%) gel in the presence of Ca^{2+} ions. Pectins with a higher DE can also form gels in this way, providing there are a sufficient number of blocks of unesterified uronic acid residues per molecule to permit the formation of a sufficient number of junction zones to form a network.[13–15] The affinity of the pectin chain for Ca^{2+}, and other cations, increases with increasing charge density along the polymer backbone and increasing 'blockiness'.[16,17] Relatively little work has been published about the interaction of pectin and organic cations, and the potential for these compounds to act as crosslinkers. It was shown[18] that a pectin (DE 68%), extracted from the cell wall material of unripe tomato formed gels on addition of poly-L-lysine, poly-L-arginine and synthetic peptides fragments of the cell wall protein extensin. In a previous study[19] we investigated the interaction between a low DE citrus pectin and poly-L-lysine. Gel formation was observed at low concentrations of crosslinker. At high levels of poly-L-lysine addition, network collapse was observed. It is of interest to see if this is a general feature of the interaction between pectin and other basic polyelectrolytes. One such polyelectrolyte is chitosan, obtained by alkaline[20] or enzyme[21] N-deacetylation of chitin to produce (1→4)-β-D-glucosamine chains. Acid hydrolysis yields low molecular weight oligosaccharides.[22] Research on pectin–chitosan films and networks,[23–25] suggests that the interaction between pectin and chitosan is attractive. In the present paper, we investigate the effectiveness of chitosan oligomers, to crosslink pectin networks, which differ in charge density and its distribution.

2. Experimental

2.1. Materials

Citrus pectins, which were chemically and enzymically de-esterified were obtained from CP Kelco. The pectins were characterised as described.[15] The fragmentation of the pectins and the subsequent analysis of the oligomeric products by high pressure anion exchange chromatography (HPAEC), to obtain the charge distribution, was carried out as described.[26] Chitosan oligosaccharide lactate was obtained from Sigma. The reported degree of deacetylation was >90%.

2.2. Analytical methods

Electrospray ionisation mass spectra (ESI-MS) of chitosan oligomers was obtained with a Micromass Quattro II mass spectrometer. The infrared spectra of films of pectin, chitosan and pectin–chitosan complexes were recorded using a Perkin–Elmer 1600 series FTIR spectrometer. Measurements of specific viscosity as a function of pectin concentration (0.01–0.1% w/w) in 50 mM acetate buffer (pH 5.6) were carried out using Ubbelohde viscometers at 20 °C. The efflux time for buffer was 115 s and no shear rate corrections were made. The intrinsic viscosity [η] was the intercept of the linear regression of reduced viscosity versus concentration extrapolated to zero concentration.

2.3. Mechanical properties of gels

~300 mg of a 3% (w/w) pectin solution in 50 mM NaOAc buffer pH 5.6 was poured into a 2 mL straight-sided (8 mm diameter) microcentrifuge tube. The pectin solution was acidified to pH ~2 by addition of 0.3 M HCl. After 30 min a known amount of 10% w/w chitosan was added. After 16 h at 4 C the pH of the mixture was adjusted to 5.5 by addition of 2 M NaOAc. Gels were removed from the microcentrifuge tubes after a further 16 h and their properties measured after thermal equilibration at room temperature. The stiffness of the gels 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.[27]

2.4. Chitosan–pectin binding behaviour

Pectin–chitosan gel (~300 mg) was immersed in 1 mL of 0.1 M NaCl for 24 h at 20 °C. The amount of free chitosan in the solution was determined by size exclusion HPLC using a BioSep-SEC-S 2000 column operating at a flow rate 1 mL/min, with 0.1 M NaCl as the eluant, and a refractive index detector. Alternatively the gel was centrifuged for 2 h at 12,500 rpm, and the supernatant collected. Chitosan in the supernatant was determined by HPLC using a Nucleosil NH_2 column operating at a flow rate of 1 mL/min, with acetonitrile/water (67:33) as the mobile phase and a refractive index detector.

3. Results and discussion

3.1. Material characterisation

The chitosan oligomer preparation was characterised by ESI-MS. A series of $[M+H]^+$ ions including monomer (180); dimer (341); trimer (502); tetramer (663); pentamer (824); and hexamer (985), were obtained corresponding to $(GlcNH_2)_n$. The oligomer ratio was obtained by reverse phase HPLC using a refractive index detector (Table 1). More than 75% w/w of the material had a degree of polymerisation between trimer and pentamer.

Table 1. Chitosan oligomer content

DP	R_t (min)	% w/w
1	7.61	2.8
2	9.48	13.2
3	11.91	26.4
4	15.17	24.1
5	19.35	25.8
6	23.13	7.7

Table 2. Physico-chemical properties and composition of pectin

Sample	Neutral sugar content (% w/w)				% GalA	% DE
	Rha	Ara	Gal	Glc		
8F	9.4	20.7	65.1	4.8	89.1	36.7
8B	8.6	28.8	56.8	5.8	87.3	57.7
40	7.6	39.7	48.4	4.3	90.3	71.2
5E	14.2	4.3	76.0	5.5	92.4	35.6
5C	10.0	7.6	78.7	3.7	91.3	51.4
5A	6.3	29.0	62.0	2.7	85.4	70.6

A range of citrus pectins was examined, the chemical characteristics of which are summarised in Table 2. All the pectins were high in galacturonic acid (85.4–92.4% w/w) with a relatively low neutral sugar content. The main neutral sugars present—rhamnose, galactose and arabinose—were typical of the pectic polysaccharides. The DE of the pectins ranged from ~36% to 71%. This range was achieved by using either chemical or enzymic de-esterification. The pattern of distribution of the uronic acid in the pectin was examined using a chemical fragmentation procedure. The methyl esterified uronic acid derivatives in a partially esterified pectin were degraded and specifically cleaved to generate a series of oligogalacturonic acid residues bearing an arabitol residue as aglycone. Chromatographic analysis of the fragments produced are shown in Figure 1. 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 Ref. 26. The structure of the major components was confirmed by negative-ion ESI-MS. In order to permit comparison of the different pectins, the traces were normalised relative to the peak area of $(GalA)_2$-1,5-di-D-Araol (retention time ~ 8 min). With decreasing DE the proportion of higher oligomers

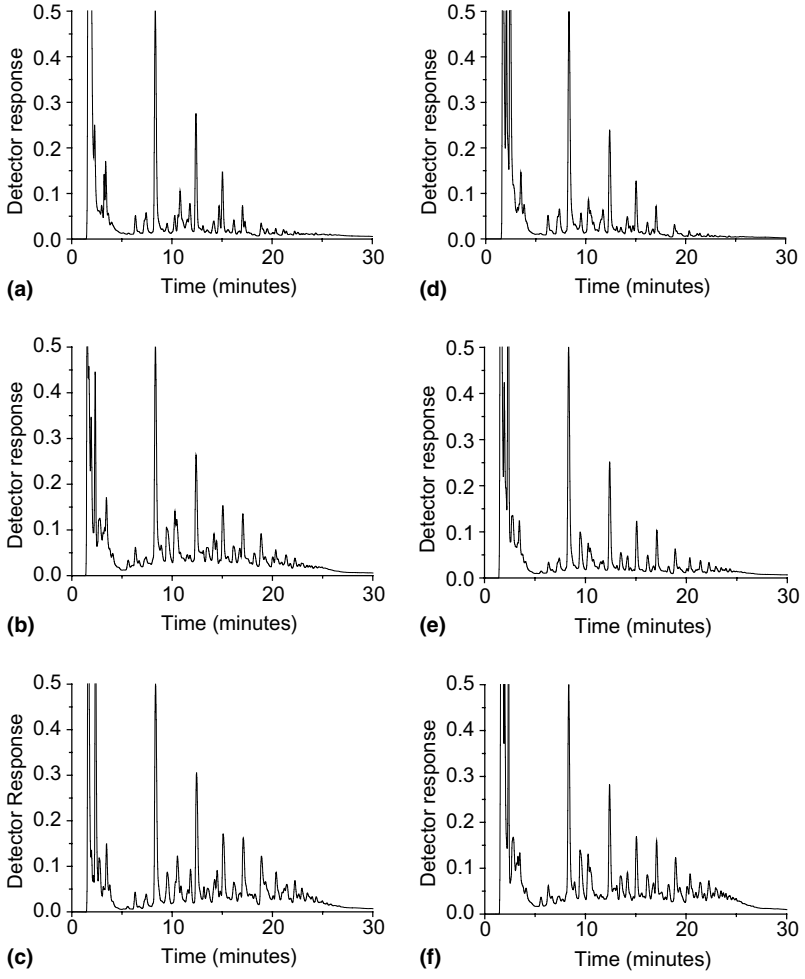

Figure 1. HPAEC at pH 6 of fragmented pectins 5A (a); 5C (b); 5E (c); 40 (d); 8B (e) and 8F (f).

increases. For pectins with a similar DE, generated using chemical (Fig. 1a–c) and enzymic approaches (Fig. 1d–f), the profile of oligomers obtained is similar.

3.2. Gel formation and properties

We investigated the effect of chitosan addition on the gelation of citrus pectins differing in charge density. Pectins with a DE of ~70% (40, enzyme de-esterified, and 5A, chemically de-esterified) did not form gels until the pectin concentration approached 5% (w/w), and the chitosan/pectin charge ratio was $\gtrsim 4.4$. The other, more highly charged pectins—8B, 8F (enzyme de-esterified), 5C, 5E (chemically de-esterified) formed gels at a pectin concentration of 3% (w/w) and at a chitosan/pectin charge ratio > 0.05. The gels behaved as elastic solids, which recovered from small static deformations. Over the range of chitosan/pectin charge ratios examined, from 0.05 to 3.38, the gels were clear, indicating that the associations that were formed were less than the wavelength of light in size.

The effect of chitosan concentration on gel stiffness for a 3% (w/w) pectin network in 50 mM NaOAc buffer pH 5.6 was examined (Fig. 2). Gel stiffness showed a strong dependence on DE of the pectin. For the pectins with a DE of ~36, (8F and 5E) the development of gel stiffness with chitosan concentration was similar at the higher concentrations of chitosan and showed a strong concentration dependence. For the more highly esterified pectins with a DE of 51–58% the development of gel stiffness with chitosan concentration showed a less marked dependence on chitosan concentration. Although it was observed that DE was the major determinant of gelation behaviour, differences were observed between the pectins, which were chemically and enzymically de-esterified. Pectins, which were de-esterified with enzyme formed gels at lower chitosan concentration compared to pectins with a similar degree of esterification, which were chemically de-esterified. The results demonstrate that the effectiveness of chitosan as crosslinking agent depends mainly on charge density.

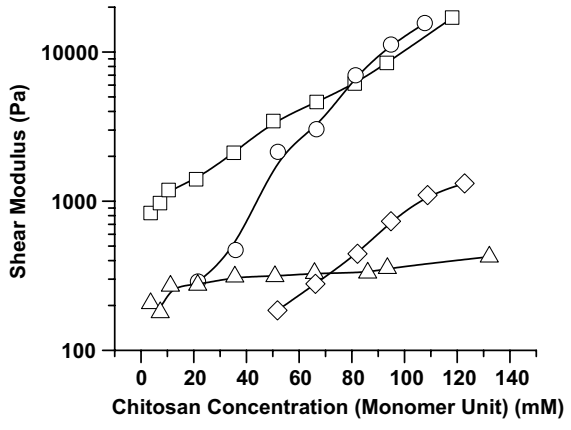

Figure 2. Dependence of shear modulus on chitosan concentration (calculated as monomer unit concentration) in pectin gels (□—pectin 8F; ○—pectin 5E; △—pectin 8B; ◇—pectin 5C), formed with a 3% w/w pectin solution in 50 mM acetate buffer at pH 5.6.

In the comparison of the gelling behaviour of pectins of different structure, it is useful to have an estimate of the number of crosslinks, which would be needed to produce a gel of a certain stiffness. This estimate can be obtained from the theory of rubber elasticity for crosslinked polymers.[28] This applies to crosslinked systems in which the mechanism of energy storage in the gel on deformation is entropic. The molecular weight between crosslinks, M_c, can be calculated from the relationship

$$G' = cRT/M_c, \quad (2)$$

where c is polymer concentration and R and T have their usual meanings. For the 3% (w/w) chitosan/pectin gels examined, M_c is typically in the range $\sim 10^4$ to 10^5 with ~50 to 500 residues between crosslinks. For polyelectrolyte gels, charge effects could lead to additional contributions to the observed elasticity, and increase this value further. The estimate indicates that relatively few crosslinks are needed to account for the observed elasticity. Consequently it is difficult, using chemical methodologies, to identify the structures, such as regions of unsubstituted galacturonic acid residues, which are responsible for this crosslinking. While the chemical fragmentation of pectins gives a useful indication of polymer charge density, it is not sufficiently sensitive to reveal the structural origin of differences in gelation behaviour in lightly crosslinked systems.

3.3. FTIR analysis of pectin, chitosan and pectin–chitosan complexes

Figure 3 shows IR spectra of pectin, chitosan and pectin–chitosan complexes. Bands at 1732 and 1644 cm^{-1} in the pectin spectrum could be attributed to esterified and nonesterified carboxyl groups, respectively.[29] The broad band at 1585 cm^{-1} in the chitosan spectrum is associated with the amine group of glucosamine,[30] the shift in this band to ~1601 cm^{-1} in the spectrum of pectin–chitosan indicates a change in environment of this group through its interaction with pectin.

3.4. Chitosan binding in chitosan–pectin gels

The binding of chitosan to pectin in chitosan/pectin gels was examined by determining the amount of free chitosan. The experimental data were plotted as a binding isotherm of $[NH_3^{4+}]_b/[COO^-]$ versus $[NH_3^+]_t/[COO^-]$, where $[NH_3^+]_b$ and $[NH_3^+]_t$ are the bound and total concentrations of glucosamine monomer, respectively, and $[COO^-]$ is the concentration of galacturonic acid monomer (Fig. 4). For the calculation it is assumed that the carboxyl and amine groups are fully dissociated.

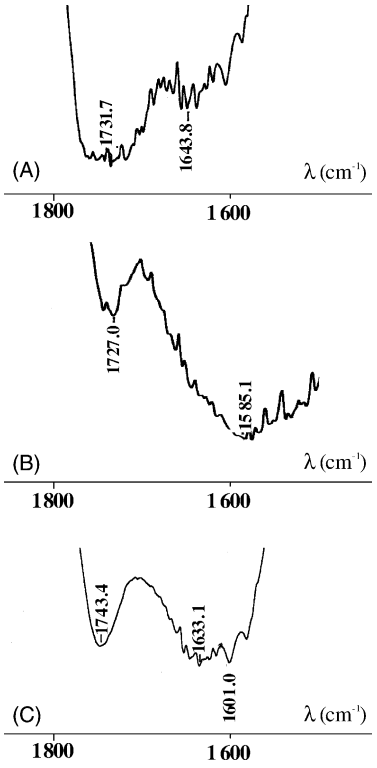

Figure 3. IR spectra of pectin (A); chitosan (B) and chitosan–pectin complex (C).

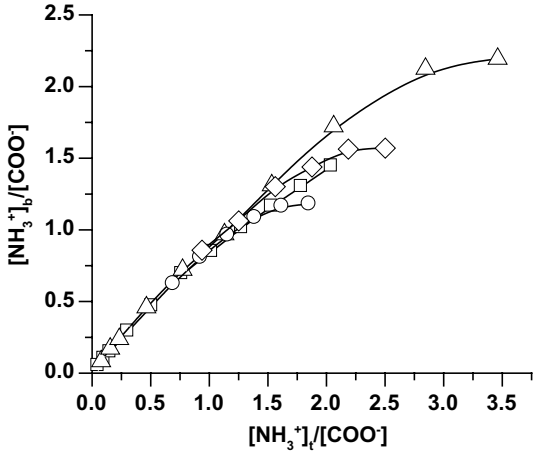

Figure 4. Binding isotherms of chitosan to 3% w/w pectin gels (□—pectin 8F; ○—pectin 5E; △—pectin 8B and ◇—pectin 5C) in 50 mM acetate buffer at pH 5.6.

At $[NH_3^+]_t/[COO^-]$ ratios < 1 the pectins behave similarly. At higher concentrations of chitosan there is only a limited tendency to show a 'saturation' in the binding behaviour. This potentially indicates that the binding of chitosan to pectin can overcharge the pectin molecule and produce a polyelectrolyte complex, which is positively charged. The binding behaviour of chitosan was analysed in more detail by using the Hill equation:[31]

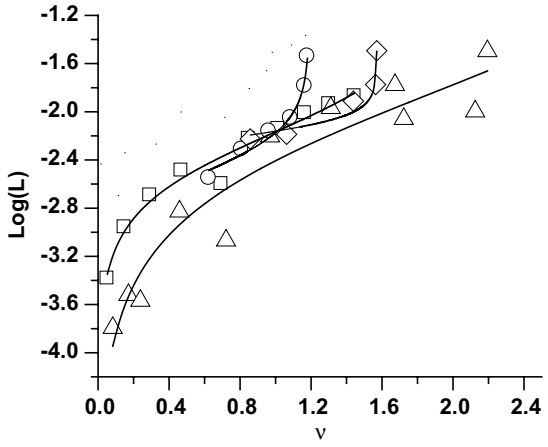

Figure 5. Hill plot of binding behaviour of chitosan to 3% w/w pectin gels (□—pectin 8F; ○—pectin 5E; △—pectin 8B and ◇—pectin 5C) in 50 mM acetate buffer at pH 5.6.

$$\mathrm{Log}\,L = -\frac{1}{\alpha_H}\mathrm{Log}\left(\frac{n_H}{v} - 1\right) + \mathrm{Log}\,K_H, \qquad (3)$$

where v is the amount of chitosan bound by a pectin segment, L the concentration of free chitosan, and n_H is the number of sites per segment able to bind cations. A pectin segment was considered to contain a single charge, in which case if the binding of the cation forms a 1:1 complex, n_H should be 1. K_H is the apparent dissociation constant for the interaction of chitosan and pectin and the reciprocal, K_B, the stability constant. α_H, the Hill constant, is an index of the cooperativity, if $\alpha_H > 1$, the binding is cooperative and when $\alpha_H < 1$, the binding is anti-cooperative. The Hill plots for chitosan are presented in Figure 5, and the derived values for α_H, n_H and K_B are shown in Table 3. With the exception of pectin 8B, the values for α_H are >1 and indicate a cooperative binding of chitosan to the pectin. These values are comparable to those obtained for the binding of Ca^{2+} to pectin.[31] α_H is larger for the chemically de-esterified pectins than the enzyme de-esterified pectin. The weakly anti-cooperative behaviour for the binding of chitosan to pectin 8B is consistent with the tendency for saturation, observed in the binding isotherm and for the weak dependence of shear modulus on chitosan concentration. The values of K_B obtained, between 83 and 316, indicate a relatively weak interaction, and are smaller than that obtained for the binding of Ca^{2+} to pectin networks ($\log K_B$ 3.9). For pectins de-esterified in

Table 3. Binding parameters for the interaction between chitosan and pectin

Sample	α_H	n_H	K_B	R^2
8F	1.37	1.96	151.4	0.97
8B	0.85	3.65	83.2	0.91
5E	2.20	1.18	316.0	0.98
5C	4.55	1.57	158.5	0.97

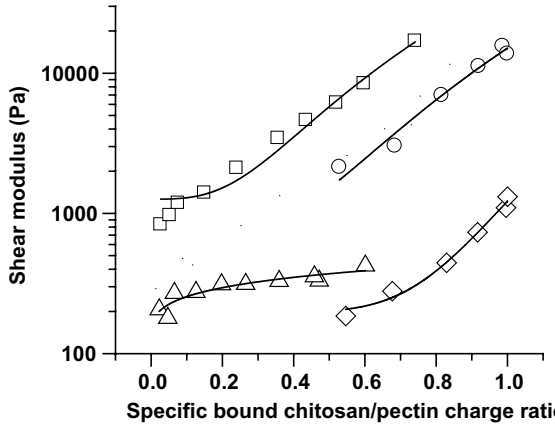

Figure 6. Dependence of shear modulus on the bound chitosan/pectin charge ratio in 3% w/w pectin gels (□—pectin 8F; ○—pectin 5E; △—pectin 8B and ◇—pectin 5C) in 50 mM acetate buffer at pH 5.6.

a particular way K_B increases with decreasing degree of esterification in agreement with previous data on the binding of inorganic cations to pectin.[16] All values for n_H are greater than 1. This observation suggests that not all the NH_2 groups present bind to carboxylate groups. One possible reason could be that there is only a partial dissociation of the NH_2 group of chitosan at pH 5.6. The pK_a of the glucosamine in chitosan is in the region of 6.5 and is dependent on ionic strength and chitosan structure. At pH 5.6 it is expected that a large fraction of the glucosamine monomer will be dissociated. An alternative explanation is that only a part of the chitosan oligomer is tethered to the pectin chain.

It is useful when comparing the gelation behaviour of different pectins to consider the effect of pectin charge on the observed behaviour. Based on the calculated values for n_H, the concentration of bound chitosan was calculated as a function of chitosan/pectin charge ratio for each of the pectins. In Figure 6, the shear modulus of chitosan/pectin gels is plotted as a function of the charge ratio of bound chitosan/pectin. The data were fitted to a power law dependence

$$G' = G'_0 + Kc^n, \quad (4)$$

where c is the bound chitosan/pectin charge ratio. The data show that over the range studied a low DE (<50%) favours gelation and that enzyme de-esterified pectin forms gels at lower concentration of crosslink.

The binding behaviour of chitosan to the gel network was further examined through the determination of the chain profile of the free chitosan in solution by HPLC. In this case, information about the binding of different oligomers can be obtained (Table 4). Over the range of degrees of polymerisation examined there is preferential binding of pentamer and hexamer, with the extent of binding decreasing with decreasing degree of polymerisation.

4. Conclusions

Chitosan can act as an effective crosslinker of pectin networks at pH 5.6. Gelation behaviour is dependent on the degree of esterification of the pectin, with pectins of a relatively low DE (36%) readily forming gels. Gel stiffness increased with increasing concentration of crosslinker. Analysis of the binding behaviour of chitosan and pectin indicated that it was possible to obtain chitosan/pectin charge ratios of bound chitosan of greater than unity. Even at these charge ratios, the gel network was clear.

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Table 4. Binding of chitosan oligomers to pectin molecule

Pectin	DE	Chitosan/pectin charge ratio	Ratio between bound and total chitosan					
			Total	Dimer	Trimer	Tetramer	Pentamer	Hexamer
5E	35.6	2.04	0.83	0.59	0.90	0.94	1.0	1
8F	36.7	2.04	0.83	0.61	0.79	0.86	0.94	0.87
5C	51.4	2.04	0.78	0.52	0.73	0.79	0.92	0.90
8B	57.7	2.04	0.81	0.64	0.79	0.84	0.99	0.80

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