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The deposition and stability of pectin/protein and pectin/poly-L-lysine/protein multilayers

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Abstract

The sequential deposition of pectin and protein – bovine serum albumin (BSA), β-lactoglobulin (BLG) and gelatin – to form multilayer structures was examined by Fourier transform infrared-attenuated total reflection spectroscopy (FTIR-ATR) and a quartz crystal microbalance with dissipation monitoring (QCMD). With each layer deposited there was a progressive increase in mass deposited, with a more substantial deposition of protein. Pectin deposition led to a relatively hydrated, open structure which permitted binding of protein within the layer when the biopolymers carried an opposite net charge. On increasing the pH, disassembly of the structures occurred within the vicinity of the isoelectric point of the globular proteins. No disassembly was observed for the pectin/gelatin multilayer. When a globular protein was substituted for a poly-L-lysine layer in a pectin/poly-L-lysine multilayer it was displaced by the subsequent deposition of a poly-L-lysine layer, the more highly charged polycation displacing the relatively low charged polyampholyte. The pectin/poly-L-lysine/protein multilayers remained intact upon titration to pH 8.0.

Keywords: Pectin; Poly-L-lysine; Bovine serum albumin; Lactoglobulin; Multilayer; Quartz crystal microbalance

1. Introduction

Protein/polysaccharide mixtures are widely encountered in nature and in industrial products. Attractive interactions between positively charged peptides/proteins and anionic polysaccharides can lead to a range of microstructures including – precipitates; gels (Macdougall et al., 2001), where a cationic peptide acts as a crosslink; coacervates (Turgeon, Beaulieu, Schmitt, & Sanchez, 2003), where the attraction of opposite charges on the biopolymers leads to liquid–liquid phase separation to produce a concentrated and dilute mixed biopolymer phase (Weinbreck, de Vries, Schrooyen, & de Kruif, 2003); and multilayers where the sequential deposition of oppositely charged polymers leads to surface crosslinking and successive charge reversal at the surface, permitting the build up of a multilayer (Decher, 1997). Proteins can also form soluble complexes with polyelectrolytes under conditions where the average net charge on the protein has the same sign as the polyelectrolyte (Hallberg & Dubin, 1998; Park, Muhoberac, Dubin, & Xia, 1992). In this case, the polyelectrolyte interacts with oppositely charged 'patches' on the protein surface allowing the formation of a soluble complex. The strength of the attraction between charged polymers depends on pH and ionic strength. The latter includes effects on the screening of attractive electrostatic interactions, and on entropic contributions to attraction such as the release of counterions on the association of oppositely charged polymeric species (Mascotti & Lohman, 1990).

Polyelectrolyte multilayers are non-equilibrium structures whose properties are strongly influenced by the conditions of their preparation. For multilayers which

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contain proteins, various types of structure can be distinguished: proteins may adsorb on the surface of preformed multilayers (Ladam et al., 2000; Ladam, Schaaf, Cuisinier, Decher, & Voegel, 2001; Laos, Parker, Moffat, Wellner, & Ring, 2006; Salloum & Schlenoff, 2004); the protein may be embedded in a mixed polyanion/polycation multilayer (Schwinte et al., 2002); or the multilayer may be fabricated by allowing the protein to replace one of the polyelectrolytes (Izumrudov, Kharlampieva, & Sukhishvili, 2005; Lvov, Ariga, Ichinose, & Kunitake, 1995). Most studies have examined the incorporation of proteins into multilayers fabricated from synthetic polyelectrolytes. These structures have potential application as enzyme reactors, or as matrices which permit the release and capture of an active component in response to small changes in environmental conditions such as pH or ionic strength.

The environmental responsiveness of the structures will depend on the charge and charge distribution within the multilayer and the ionic environment. For structures fabricated from a weak polyelectrolyte and a polyampholyte (protein) the charge and its distribution will show a marked dependence on pH. In this article, we examine the assembly and environmental responsiveness of biopolymer multilayer structures where the protein either replaces a biopolycation in the multilayer, or is embedded in a biopolyanion/biopolycation multilayer. The proteins we have chosen for this study are the globular proteins bovine serum albumin (BSA) and β-lactoglobulin (BLG), and a flexible protein – gelatin. For the biopolyanion we have chosen pectin which is a weak polyelectrolyte based on chains containing linear regions of (1→4)-α-D-galacturonosyl units, with a fraction of the uronic acid residues being methyl esterified. The work complements our previous study of pectin/PLL multilayer assembly (Krzeminski et al., 2006) by replacing the PLL polycation either wholly or partially with a protein polyampholyte.

2. Materials and methods

2.1. Polyelectrolyte solutions

Poly-L-lysine hydrobromide (PLL) with a mean degree of polymerization of 70, bovine serum albumin, and β-lactoglobulin were obtained from Sigma, an acid-extracted gelatin (isoelectric point 8.3) was obtained from Rousellot (Hungerford, UK), citrus pectin with a degree of esterification of 51% was obtained from CP Kelco. D_2O (99.9%) was obtained from Sigma. Reagents were analytical grade. For multilayer fabrication, 0.6 mg mL^{-1} solutions of the proteins and pectin were prepared in 10 mM buffer containing 30 mM NaCl, in the case of gelatin initial dissolution was carried out at 80 °C. 0.08 mg mL^{-1} PLL solutions were prepared in the same buffer. Acetate and phosphate buffers were used for the multilayer fabrication and responsiveness over the pH range 3.6–8.0, as appropriate, each containing 30 mM NaCl.

2.2. Fourier transform infrared-attenuated total reflection spectroscopy (FTIR-ATR)

Infrared spectra were collected, over the range 2000–1500 cm^{-1}, on a Nicolet 860 FTIR spectrometer (Thermo Electron Corporation, Madison, USA) fitted with a Micro-Circle liquid ATR cell (SpectraTech, Warrington, UK). The ATR crystal was a silicon cylinder (Thermo Electron, Madison, USA) mounted in a thermostatted steel jacket controlled at a temperature of 20 °C. Solution spectra of PLL, pectin and the proteins in D_2O were determined as a function of concentration over the concentration range 0–10 mg mL^{-1}. The peak heights of the characteristic amide I absorbances of PLL (Jackson, Haris, & Chapman, 1989), BLG (Dong et al., 1996), BSA (Sukhishvili & Granick, 1999) and gelatin in the region of 1645 cm^{-1}, and pectin (Monsoor, Kalapathy, & Proctor, 2001a, 2001b) carboxylate (1610 cm^{-1}) and ester (1743 cm^{-1}) showed a linear dependence on concentration with a small positive intercept at zero concentration which was indicative of adsorbed material at the crystal surface. FTIR-ATR of multilayer structures, and the analysis of the spectra to obtain the mass of material deposited was carried out as described (Krzeminski et al., 2006). Multilayers were built up by first depositing a poly-L-lysine base layer, followed by a buffer wash, then a pectin layer, followed by a buffer wash, then a protein layer, and so on, until the required number of layers had been deposited. Absorbances were measured relative to a baseline at 1800 cm^{-1}, a frequency at which there was no absorbance in the present systems. The amounts of pectin and protein deposited in each layer of the multilayers was estimated by subtraction of spectra to obtain their difference and calculation of the amount adsorbed using pectin and protein calibration curves and the penetration depth. The penetration depth calculated, as previously (Krzeminski et al., 2006; Sperline, Muralidharan, & Freiser, 1987), assuming a silicon refractive index of 3.42, varied from 452 to 491 nm over the frequency range 1743–1605 cm^{-1}. During multilayer assembly material was irreversibly adsorbed after buffer washing and so the cumulative mass could be obtained by summing the washed layer masses.

2.3. Quartz crystal microbalance

Measurements were carried out using a D300 quartz crystal microbalance with dissipation monitoring (QCMD) (Q-Sense AB, Västra Frölunda, Sweden) with a QAFC 302 axial flow measurement chamber as described (Krzeminski et al., 2006). The sensing element is a disc-shaped, AT-cut piezoelectric quartz crystal sandwiched between two gold electrodes. The crystal is excited to oscillation at its fundamental resonant frequency (~5 MHz). A small mass deposited (Δm) on the gold sensing surface will cause a decrease in resonant frequency (Δf_n). If the mass is deposited evenly and is sufficiently rigid then the mass adsorbed is directly

proportional to the change in frequency according to the Sauerbrey equation (Sauerbrey, 1959)

$$\Delta m = -C\Delta f_n/n \quad (1)$$

where C is the mass sensitivity constant ($C = 17.7$ ng cm^{-2} Hz^{-1} for a 5 MHz crystal (Hook, 2001)) and n is the overtone number ($n = 1, 3, 5\ldots$). For viscoelastic layers a more detailed analysis using a Voigt viscoelastic model fitted with the QTools™ modeling package (Q-Sense AB, as above) was used as described (Krzeminski et al., 2006).

The formation of multilayers was investigated at 20 °C as described (Krzeminski et al., 2006). Multilayers were built up in a way similar to the FTIR-ATR experiment though, in contrast, the QCMD allowed continuous collection of data. Between each experiment the crystal chip was cleaned by soaking in 2% Hellmanex solution (Hellma UK Ltd., Southend, UK) for 15 min. The chips were then rinsed in distilled water, dried with N_2, placed in an ultraviolet cleaning chamber (BioForce Nanosciences, Ames, IA, USA) for 15 min, rinsed and dried as before. The measurement chamber was rinsed with 5 mL 2% Hellmanex solution followed by 80 mL of water.

3. Results

3.1. Assembly of pectin–protein multilayers

Multilayer assembly on silica surfaces was examined using FTIR-ATR. Silica acquires a negative surface charge through the dissociation of silanol groups, which is dependent on local chemical environment. Literature values of the pK_a and site density of silanol groups are 7.5 and 8 nm^{-2}, respectively (Behrens & Grier, 2001). Although it is expected that the surface charge would fall to zero at acid pH, electroosmotic mobility experiments show that the silica surface can retain a small negative charge in the pH range 2–4 (Lambert & Middleton, 1990; Thormann, Caslavska, & Mosher, 1995).

The initial fabrication of 8-layer multilayers was studied. Fig. 1 shows the FTIR response on deposition of a PLL base layer (layer 1) followed by successive deposition of pectin and protein layers for BSA (b), BLG (c), and gelatin (d) at pD 3.6. The solution spectra of the proteins (Fig. 1a) shows a strong absorbance characteristic of amide I at ~1645 cm^{-1}, and a weaker absorbance in the region normally associated with amide II in water at 1550 cm^{-1}. The conversion of NH to ND, through deuterium exchange, shifts the amide II band to a lower wavenumber (Sukhishvili & Granick, 1999) in the region of 1450 cm^{-1}. For human serum albumin at pD 9.2, the remaining absorbance in the vicinity of 1575 cm^{-1} was attributed to the stretching vibrations of the carboxylate groups of aspartate and glutamate residues with components at 1584, 1574 and 1565 cm^{-1}. For BLG at pD 7.0 a similar shift in the position of the amide II band is observed (Dong et al., 1996) after deuterium exchange with the remaining weak feature at 1572 cm^{-1} associated with acidic amino acid residues, as

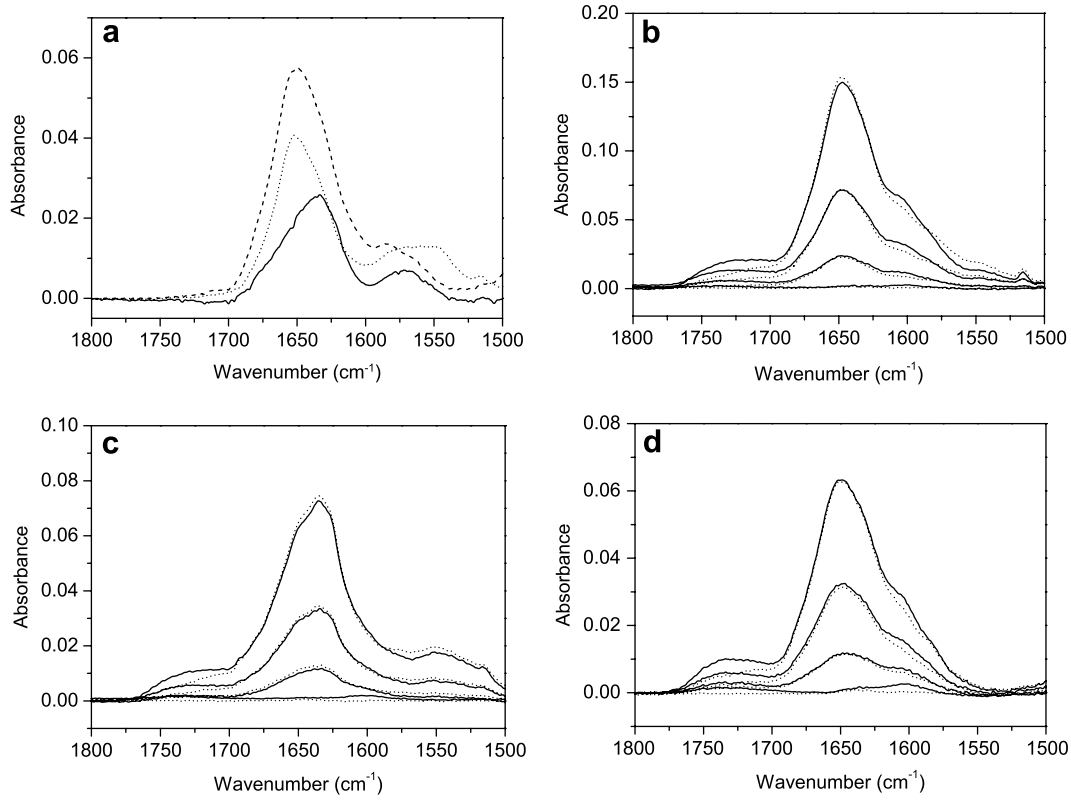

Fig. 1. FTIR spectra of the proteins in solution (a) (BSA (···); BLG (—); gelatin (---)); and for multilayers formed by the successive deposition of pectin (—) and protein (including the PLL base layer) (···) for BSA (b), BLG (c), and gelatin (d) at pD 3.6.

before. At pD 3.6 the —COOD stretch of aspartic and glutamic acid side chains occurs in the region of 1700–1750 cm^{-1}. The spectra obtained on sequential deposition of pectin and protein to a PLL base layer are dominated by the protein component. Deposition of protein results in a marked increase in the absorbance associated with the amide I band characteristic of the protein. By comparison, deposition of pectin results in relatively minor spectral changes. As the intrinsic pK_a of the uronic acid of pectin varies in the range 3.5–4.5 (Ralet, Dronnet, Buchholt, & Thibault, 2001) at the pD used for multilayer fabrication of 3.6, the pectin spectra potentially contains contributions associated with the C=O stretching vibration of the uronic acid methyl ester (Monsoor et al., 2001b) at ~1743 cm^{-1}, the —COOD stretch vibrations at ~1730 cm^{-1} (these first two peaks overlap), and the asymmetric stretching of $—COO^-$ at ~1607–1617 cm^{-1}.

Deposition of pectin at pD 3.6 results in relatively small increases in absorbance in the region of 1700–1750 cm^{-1} and in the region of 1610 cm^{-1}. While these spectral changes are characteristic of pectin deposition, the amount deposited is relatively small. From the absorbance of the amide I it is possible to estimate the amount of protein adsorbed as each protein layer is deposited. Results for the final protein layer, layer 7, are presented in Table 1. The amounts of globular protein adsorbed in the multilayer (940, 1400 ng cm^{-2}) are comparable with the amounts of thermally aggregated globular protein which adsorb on oil–water interfaces (1000–1500 ng cm^{-2}) (Walstra, 2003). This is an indication of the extent to which the globular protein is bound throughout an extended pectin layer. In contrast the amount of gelatin adsorbed (560 ng cm^{-2}) is more typical for the amounts of non-globular protein adsorbed at oil–water interfaces. This indicates a lack of interpenetration of the gelatin with the pectin layer. Estimates of the pectin content were also obtained from the difference spectra using the absorbances characteristic of pectin ester and carboxyl and an estimated degree of ionization of 0.48 (Table 1). This degree of ionization was estimated from the absorbances of the ester, carboxyl and carboxylate regions of the spectrum for the completed BSA, BLG and gelatin multilayers and was found to be independent of the identity of the protein. For the pectin/protein multilayers the mass ratio of pectin to protein in the multilayer, Pe/Pr, oscillates as layer deposition proceeds from about 0.4 to 0.2 for BSA, 0.3 to 0.15 for BLG and 0.5 to 0.3 for gelatin during the sequential deposition. The multilayers are all richer in protein than pectin and to a greater extent for the globular proteins as compared with the flexible protein, gelatin. The presence of pectin was necessary for the binding of the amounts of protein observed (e.g. 1400 ng cm^{-2} BSA, layer 7). We found that the binding of the proteins to the bare surfaces was an order of magnitude lower, with a binding of <130 ng cm^{-2} for BSA (about 30% of close-packed coverage).

A condition of charge balance has been found in some polyelectrolyte multilayers (Schlenoff, Ly, & Li, 1998)

Table 1
Multilayer composition as characterised using FTIR-ATR and QCMD: protein and pectin adsorbed layer masses measured by FTIR-ATR; the overall pectin/protein (Pe/Pr) mass ratio; the anionic/cationic (−/+) charge ratio; the cumulative multilayer mass; the hydrated mass by QCMD; and the multilayer solids concentration as the final three layers are added to pectin/protein multilayers

Multilayer	Layer number (Ln) and composition	FTIR-ATR layer mass (ng cm^{-2})	Mass ratio (Pe/Pr)	Charge ratio (−/+)	Cumulative mass (ng cm^{-2})	QCMD multilayer mass (ng cm^{-2})	Multilayer solids conc. (% w/w)
Pectin/BSA	L6, pectin	280	0.43	0.70	1600	4400	37.0
	L7, BSA	1400	0.19	0.31	3000	7000	43.0
	L8, pectin	450	0.37	0.61	3500	8600	40.0
Pectin/BLG	L6, pectin	130	0.32	0.69	1100	3400	32.0
	L7, BLG	940	0.15	0.32	2000	4500	45.0
	L8, pectin	200	0.26	0.56	2200	5500	40.0
Pectin/gelatin	L6, pectin	180	0.58	–	900	6300	15.0
	L7, gelatin	560	0.29	–	1500	9800	15.0
	L8, pectin	270	0.53	–	1700	11,000	16.0

The masses of lower layers (L1–L5) are used to calculate the overall mass ratio; the charge ratio; and the cumulative mass.

typically those comprising flexible homopolyelectrolytes (Decher, 2003). If there is a balance between the cationic and anionic charges on the polymers in the multilayer (''intrinsic charge balance'') this will determine the stoichiometry of the multilayer i.e. the relative amounts of protein and pectin. At pH 3.6 the charge on BSA (Tanford, Swanson, & Shore, 1955), in elementary protonic charges, is ~48, and for a BLG monomer (Cannan, Palmer, & Kibrick, 1942) at the same pH it is ~10. For pectin with a degree of esterification of 50% and a carboxyl degree of ionization of 0.5, intrinsic charge balance would be obtained at pectin/protein mass ratios of about 0.61 and 0.46 for BSA and BLG, respectively. These estimates would require revision if there was any subfractionation of the pectin population on deposition, or if the local environment of the multilayer shifted the pK_a of the weakly acidic carboxyl group of the anhydrogalacturonosyl, aspartic acid and glutamic acid residues. In this study, the overlap of the ester and carboxyl absorption peaks in the FTIR spectrum mean that these factors cannot both be determined and in our analysis we assumed there was no subfractionation of the pectin and determined the apparent dissociation of the carboxyl groups. While other factors must clearly influence the interaction between the proteins and the pectin, including the way that the charge is distributed on the respective polymers and steric considerations, this crude calculation indicates that if the deposition of the multilayer was dominated by electrostatic interactions so that intrinsic polymer charge balance (Schlenoff et al., 1998) was achieved, then it would be expected that pectin could have the potential to adsorb substantial amounts of protein. The experimentally observed pectin/protein

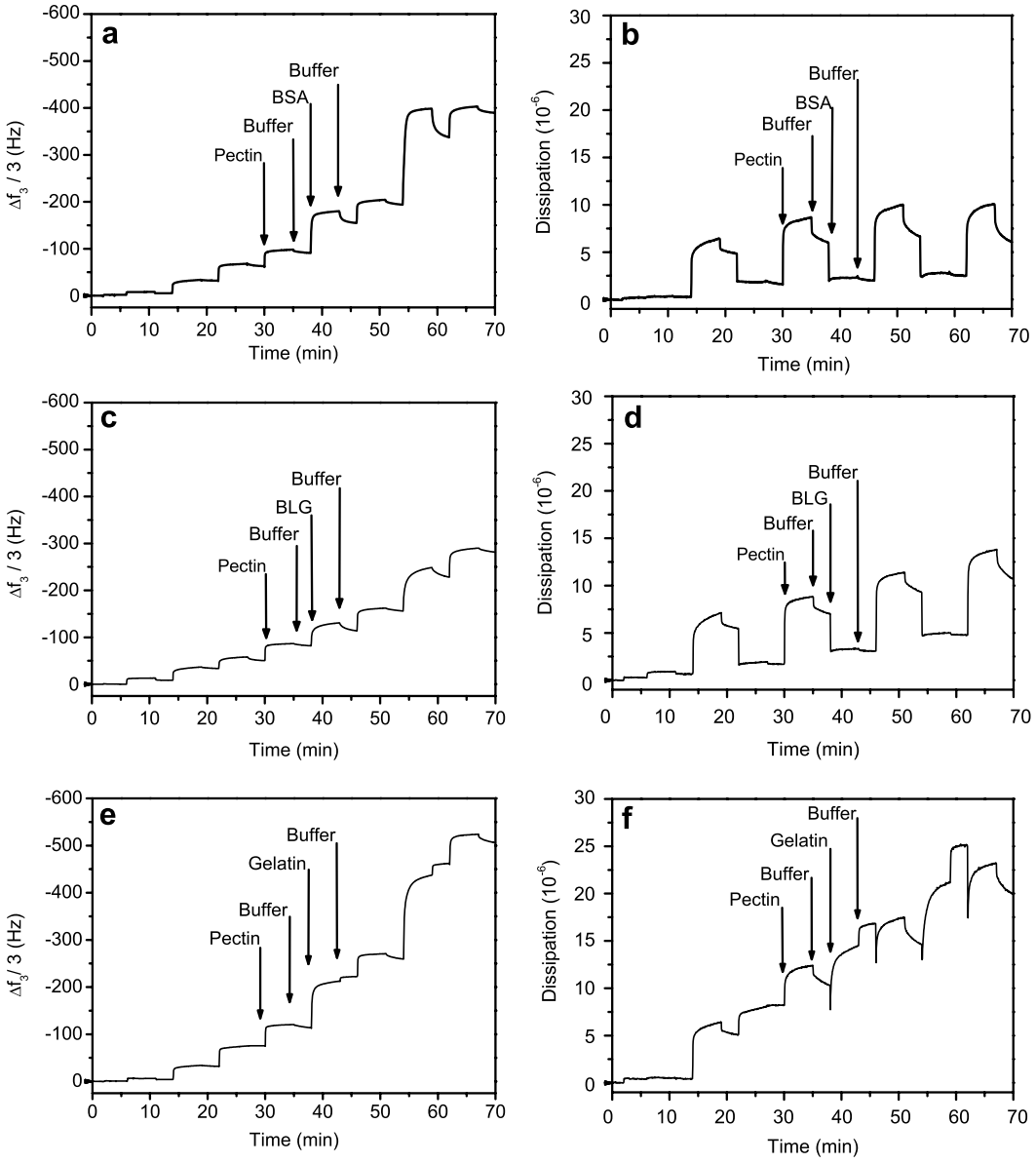

Fig. 2. QCMD frequency and dissipation shifts during the successive deposition of pectin and protein for the proteins: BSA (a and b); BLG (c and d); and gelatin (e and f). PLL base layer deposited first, before initial pectin layer, and buffer washes between each biopolymer deposition.

mass ratios suggest that the charge ratios (anionic charge/cationic charge) are less than unity and the polymers in the multilayer carry a net positive charge (Table 1). Lack of intrinsic polymer charge balance has been reported for synthetic polymer multilayers (Hoogeveen, Stuart, Fleer, & Bohmer, 1996; Riegler & Essler, 2002) and means that, to achieve charge balance, the net polymer charge must be compensated by small ions (extrinsic charge compensation (Schlenoff et al., 1998)). While Hoogeveen et al. (1996) suggested lack of intrinsic charge balance could originate from differences of charge accessibility between mainchain (e.g. pectin galacturonosyl residues) and sidechain (e.g. poly-L-lysine) charges, it has also been taken as an indication that non-electrostatic (e.g. hydrophobic) interactions are influencing multilayer assembly (Dubas & Schlenoff, 1999).

The assembly process was also followed using a quartz crystal microbalance with dissipation monitoring, which gives information on the hydrated mass of polymer deposited, and on the viscoelastic properties of the deposited layer through the measurement of the frequency dependence and dissipation (Voinova, Rodahl, Jonson, & Kasemo, 1999). Fig. 2a, c and e shows the frequency change as a function of time for the sequential deposition of pectin and protein to a PLL base layer for BSA, BLG and gelatin, respectively. In Fig. 2b, d and f are shown the corresponding changes in dissipation. With each successive deposition step of pectin or protein there is an increase in magnitude of the negative change in frequency. The relative response observed on pectin deposition is larger than that observed by FTIR, consistent with the pectin forming a relatively hydrated layer. For the pectin/globular protein multilayers, the deposition of pectin is largely irreversible over the timescales examined with a minor decrease in the frequency change on buffer washing. In contrast some of the adsorbed globular protein is removed on buffer washing at pH 3.6 indicating that a proportion of the protein interacts weakly with the multilayer. For the pectin layers containing gelatin the situation is reversed, with the deposition of gelatin being essentially irreversible, with some of the adsorbed pectin being removed on rinsing with buffer. It should be noted that at this temperature and dilution the gelatin is in a helical form (below its coil–helix transition) and will be a mixture of intramolecular and intermolecular helical structures (Harrington & Rao, 1970). Analysis of the combined FTIR-ATR and QCMD data gives an estimate of the overall solids content of the multilayers. Results are tabulated in Table 1 for the multilayers as the final three layers are added. The hydrated mass of the multilayers calculated using the Voigt viscoelastic model are 5–20% greater than the Sauerbrey mass (from Δf_3) for BSA and BLG and 20–35% greater for gelatin. For the globular proteins the oscillations in mass ratio of pectin and protein deposited are becoming smoothed out as the 6th, 7th and 8th layers are deposited, yielding multilayers with solids concentrations in the range 32.0–45.0% w/w. In contrast, the solids concentration remains essentially constant on deposition of the gelatin (layer 7) but increases as each pectin layer is deposited reaching 16.0% w/w after 8 layers, the flexible protein yielding a more hydrated structure than the globular protein. It should be noted that in making these calculations we have combined data from techniques which do not have identical sensing surfaces, whereas, the silicon FTIR crystal has a silica surface, the quartz crystal chips we used presents a gold surface. However, as multilayers are built up the effect of the base surface upon the structure progressively diminishes until a point where the growth is unaffected by the base surface (Decher, 2003). While the initial layers may differ as the number of layers increases any differences between surfaces become negligible.

The observed changes in dissipation (Fig. 2d–f) also reveal the different behaviour of pectin and proteins in the different structures, the interpretation aided by the use of the Voigt viscoelastic model (Voinova et al., 1999). On deposition of a pectin layer an increase in dissipation is observed, indicating the deposition of a relatively hydrated viscoelastic layer. On initial deposition of the globular proteins, BSA and BLG, the dissipation falls, suggesting that the globular proteins diffuse into and 'crosslink' the pectin layer. For the structure containing gelatin, deposition of the gelatin layer results in an increase in dissipation, while the addition of pectin results in a small decrease in dissipation, indicating that in this system the pectin is acting as the crosslinker. Quantitative analysis (Table 1) showed that when pectin is added to gelatin there is no increase in hydrated mass but an increase in solids content.

3.2. Multilayer stability in response to pH change

The data on the sequential deposition of pectin and proteins suggest that under the conditions used, there is a net attraction between the proteins and pectin. This could involve the attraction of oppositely charged residues on the pectin and protein, but might also involve other interactions. For example, the methyl esterified uronic acid residues of pectin have the potential to interact with proteins through a hydrophobic effect. In order to explore more

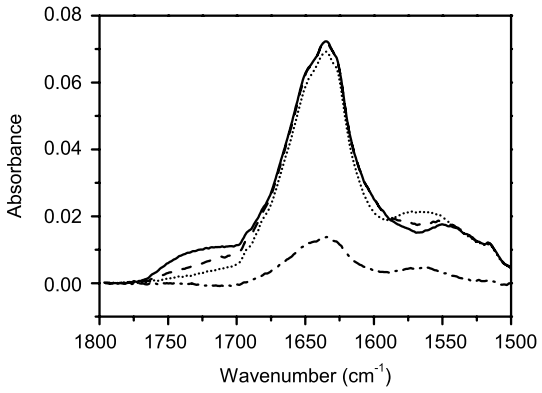

Fig. 3. FTIR spectra of a pectin/BLG multilayer at pD: 3.6 (—); 4.2 (- - -); 4.8 (· · ·) and 5.4 (— · — · —).

fully the attractive interaction between pectin and proteins, we have examined the stability of the deposited layers to changes in pH using FTIR-ATR and QCMD. Increasing pH will cause weakly acidic carboxyl groups on the pectin and protein to dissociate thus changing the charge interactions in the multilayer. Fig. 3 shows the observed spectra at pH 3.6, 4.2, 4.8 and 5.4 for the pectin/BLG multilayer. Initially, over the range pH 3.6–4.8, the absorbance associated with the carboxyl group of pectin and/or protein side chains (1730 cm^{-1}) shows a small decrease. There is also a small decrease in the absorbance associated with amide I (1635 cm^{-1}) and an increase in the absorbance associated with the aspartate and glutamate residues of the protein (1575 cm^{-1}). This indicates that while the increase in charge on the protein with increasing pH is apparent this is also associated with a limited solubilisation, particularly of protein. For the structures containing BLG and BSA, on further increase in pH there is a progressive decrease in the absorbance associated with the amide I band (Figs. 3 and 4a and c). The absorbance shows a marked change over a pH range of 1–2 units. The midpoint of this change is ~5.5 and 5.1 for BSA and BLG, respectively. As the isoionic point of the isolated proteins is ~5.3 for defatted BSA (Tanford et al., 1955) and ~5.1 for BLG (Cannan et al., 1942) this disassembly is occurring in the region of the isoelectric point of the proteins, suggesting that charge interactions play a major role in stabilizing the deposited structures, with the overall net charge on the protein

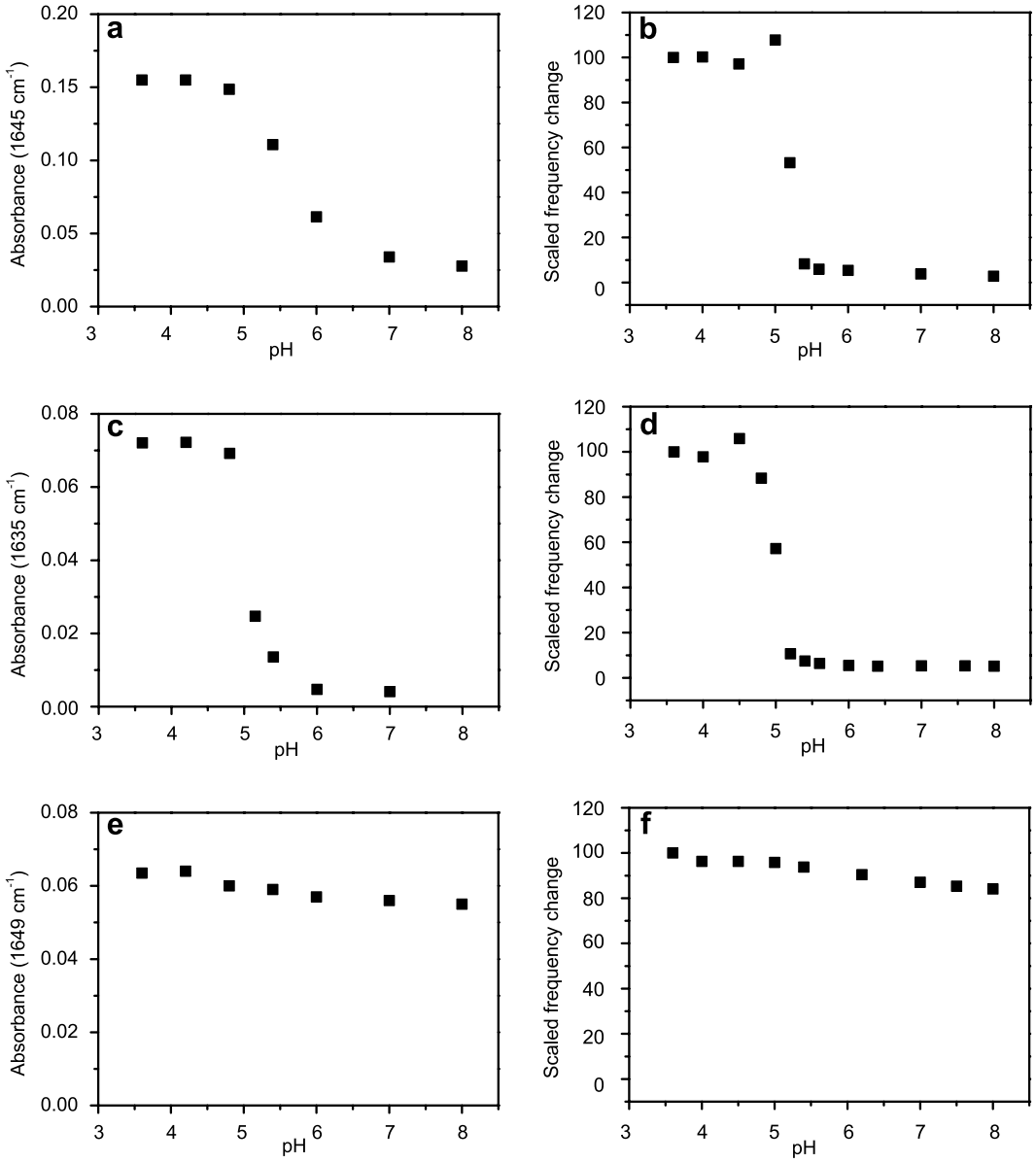

Fig. 4. Infrared amide I peak absorbance versus pH (a, c, and e) and scaled QCMD frequency change (Δf_3) versus pH (b, d, and f) for pectin/protein multilayers containing BSA (a and b), BLG (c and d), and gelatin (e and f). Gelatin QCMD results are scaled using buffer baseline prior to multilayer assembly rather than final frequency change after disassembly.

having a major influence on stability. The acid-extracted gelatin has a higher isoelectric point of 8.3 and over the pH range examined of 3.6–8.0 the absorbance of the pectin/gelatin structure in the amide I region shows little change (Fig. 4e).

The disassembly of the pectin/protein multilayers was also followed using QCMD. In Fig. 4b and d is shown the scaled frequency change at 15 MHz as a function of pH for the layers containing BSA and BLG, respectively. To emphasize the step change, the results have been scaled so that the initial and final points are 100 and 0, respectively. There is a marked fall in the vicinity of pH 5.3 and 5.1 for BSA and BLG, respectively, associated with disassembly of the layer. Immediately prior to the disassembly there is a small increase in frequency change consistent with an increase in hydrated mass of the layer, which could be due to a reduction in crosslinking and/or an increase in the driving force for swelling. The results for gelatin (Fig. 4f) showed no disassembly effect in the pH range studied, a result consistent with that observed by FTIR (Fig. 4e).

3.3. Protein incorporation into pectin/PLL multilayers

In previous research we have shown that PLL can crosslink pectin gels (Marudova, MacDougall, & Ring, 2004) and can function as the polycationic layer in pectin-containing polyelectrolyte multilayers (Krzeminski et al., 2006). The interaction of the proteins with pectin/PLL multilayers was examined using both FTIR-ATR and QCMD. Initially PLL and pectin were sequentially deposited on a silica surface to form a 4 layer base at pD 3.6. Subsequently, a single protein (BSA, BLG, gelatin) layer was deposited followed by sequential deposition of pectin, PLL and pectin to complete 8 layers, as in the previous section. Fig. 5 shows the observed spectral changes in the range 1900–1500 cm^{-1} for the deposition on the 4 layer base. The base layer has absorbances associated with pectin, including the —COOD stretch vibrations at ~1730 cm^{-1}, the C=O stretching vibration of the uronic acid methyl ester (Monsoor et al., 2001b) at ~1743 cm^{-1} and the asymmetric stretching of COO^- at ~1607–1617 cm^{-1}, and the poly-L-lysine amide I band in a random coil conformation at 1643–1648 cm^{-1} (Jackson et al., 1989). Addition of BSA (Fig. 5a), BLG (Fig. 5b) or gelatin (Fig. 5c) to this base layer results in a marked enhancement of the amide I band (layer 5 in inset). Further deposition of pectin (layer 6) results in increases in bands associated with carboxylate, carboxyl and ester groups. For both the globular proteins subsequent addition of PLL (layer 7) results in a marked change to the spectrum, the most dramatic of which is a fall in the absorbance associated with the amide I band, and an increase in the absorbance associated with the carboxylate group of pectin. In contrast, for gelatin the deposition of PLL (layer 7) causes a relatively small reduction in the amide I absorbance. For all the proteins further deposition of pectin (layer 8) results in an increase in absorbances associated with pectin, more particularly

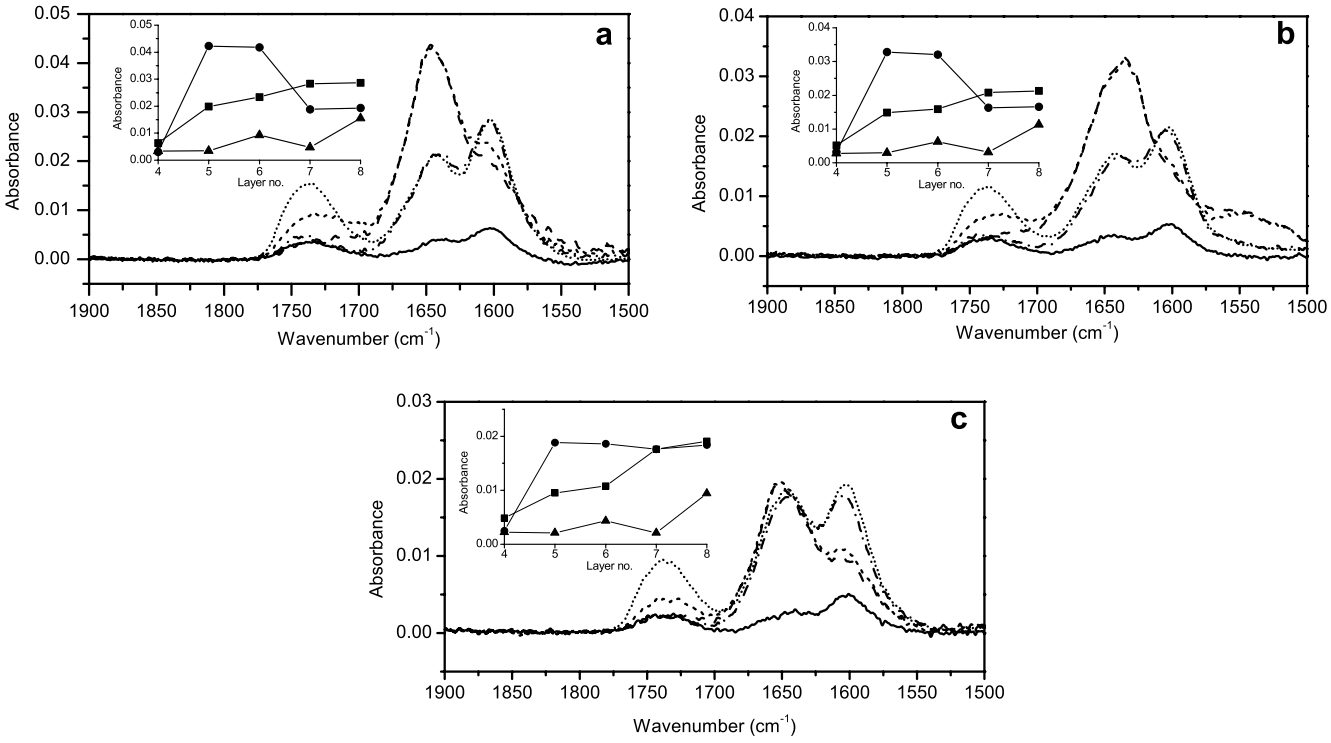

Fig. 5. FTIR spectra for a PLL/pectin/PLL/pectin multilayer (—) on which is deposited BSA (a), BLG (b), and gelatin (c) (— — —), followed by sequential deposition of pectin (---); PLL (—·—·—); and pectin (···). Insets show absorbance variation at frequencies for amide I (1645 cm^{-1}, ●), carboxylate (1610 cm^{-1}, ■), carboxyl/ester region (1730 cm^{-1}, ▲).

pectin carboxyl/ester with relatively little increase in the absorbance associated with pectin carboxylate. Although the main effect of PLL deposition is globular protein solubilisation, a secondary effect is a shift in the ionic equilibria within the layered structure, with changes in carboxyl to carboxylate ratio being observed on deposition of PLL, though subsequent deposition of pectin is predominantly in the carboxyl form, an effect which occurs for all the proteins. These perturbations and oscillations in ionic equilibria are characteristic of multilayers which contain weak polyelectrolytes. It is well established that the ionization of charged residues is sensitive to local environment, as for example in the binding of charged ligands to proteins (Antosiewicz, McCammon, & Gilson, 1996). For multilayers fabricated from weak polyelectrolytes, the dissociation constants of the polyacid and polybase in the multilayer decreased and increased, respectively, relative to that solution (Burke & Barrett, 2003), for a weak polyanion embedded in a multilayer of strong (Xie & Granick, 2002) or weak (Kharlampieva & Sukhishvili, 2003) polyelectrolytes, the ionization of the polyanion increased when a polycation formed the uppermost layer, whereas there was a reduced ionization (compared to the solution case) when a polyanion formed the uppermost layer (Xie & Granick, 2002). The range of the effect was over a relatively large length scale, exceeding the solution Debye screening length. In the current study, the structures which contain PLL are showing a similar sensitivity to local environment.

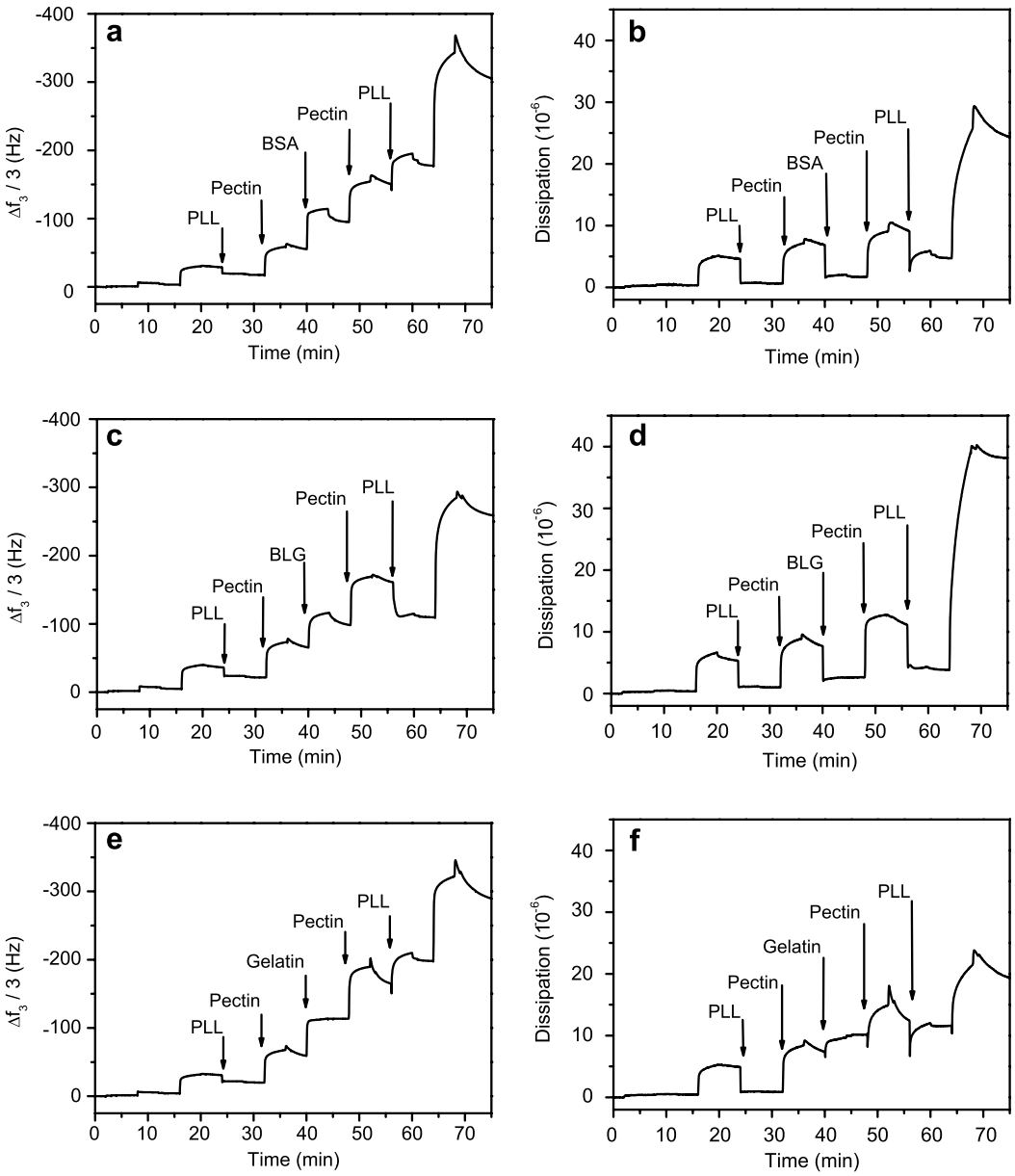

Fig. 6. QCMD frequency and dissipation shifts for the successive deposition of PLL; pectin; PLL; pectin; protein; pectin; PLL; pectin for the proteins: BSA (a and b); BLG (c and d); and gelatin (e and f).

However, the origin of the effect could be due to differences in the local complexation of the pectin with the protein and PLL (Wen & Dubin, 1997). In both situations the addition of the polycation (PLL, layer 7) resulted in an apparent pK_a decrease of the polyanion (pectin).

Quantitatively the final amide I absorbance for the pectin/PLL/protein multilayers (Fig. 5) is only 30% of that of the pectin/protein multilayers (Fig. 1) indicating significantly less PLL has been deposited as compared with the proteins. Further quantitative analysis of multilayer composition was not tractable due to the changing ionization of the pectin introducing an additional variable.

The changes in hydrated mass on deposition of this structure were also followed by QCMD. Several general features can be noted on examination of the frequency change on deposition (Fig. 6). Deposition of pectin results in a progressive increase in frequency change associated with a mass increase. A relatively larger response is observed in QCMD compared to FTIR-ATR, suggesting that the pectin is deposited as a relatively hydrated layer. Initial addition of PLL (layer 3) results in a frequency change associated with hydrated mass loss, this characteristic change on PLL deposition was observed previously and occurs as a result of two factors. The crosslinking of the pectin layer with PLL, and the deswelling of this layer through the replacement of monovalent cations, with a polyvalent cation leading to a reduced polyelectrolyte swelling effect (Krzeminski et al., 2006). In contrast to PLL, the deposition of the protein layer (layer 5) results in an increase in frequency shift, which once viscoelasticity is taken into account, corresponds to the swelling of the multilayer for BSA and gelatin and the multilayer hydrated mass remaining constant for BLG. The multilayer with BLG deposited shows distinct behaviour on the subsequent addition of PLL to the pectin layer (layer 7), with the frequency change (after buffer washing) decreasing which resulted from a 30% loss of hydrated mass. This behaviour is typical of a pectin/PLL multilayer and combining this with the interpretation of the FTIR-ATR suggests that the BLG has been extensively displaced from the multilayer as it shrinks due to PLL crosslinking. The shrinkage does not occur for the BSA multilayer perhaps due to higher retention of the larger globular protein. The multilayer with gelatin deposited shows an increase in hydrated mass on deposition of the final PLL layer (layer 7), its hydrated structure showing no tendency to shrink on crosslinking.

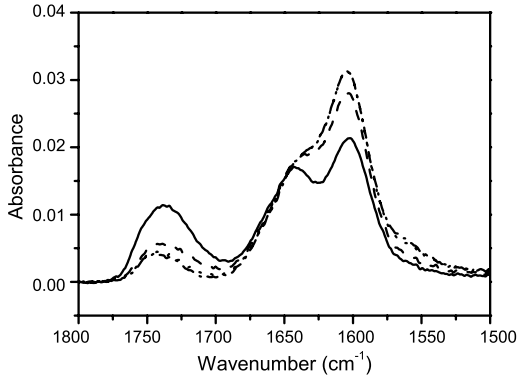

Fig. 7. FTIR spectra of pectin/PLL/BLG multilayer at pD: 3.6 (—); 4.2 (---); 4.8 (···); and 5.4 (–·–·–).

3.4. Stability as a function of pH

The responsiveness of the assembled pectin/PLL/protein multilayers to change in pH, as probed using FTIR-ATR, were similar for the different proteins and the result for the BLG containing multilayer is shown in Fig. 7. There is an increase in the absorbance of the carboxylate and a corresponding reduction in absorbance of the carboxyl over a pH range which is consistent with the solution pK_a of the pectin. The variation of carboxylate absorbance is shown for all three proteins in Fig. 8a. In each case the absorbance passes through a maximum, consistent with ionization at pHs less than 5.4, and limited pectin solubilisation at higher pH. In the QCMD experiment a frequency change associated with increase of hydrated mass was observed for BSA and gelatin (Fig. 8b). In contrast the multilayer in which BLG was deposited showed little response to pH change. Whereas pectin/protein multilayers

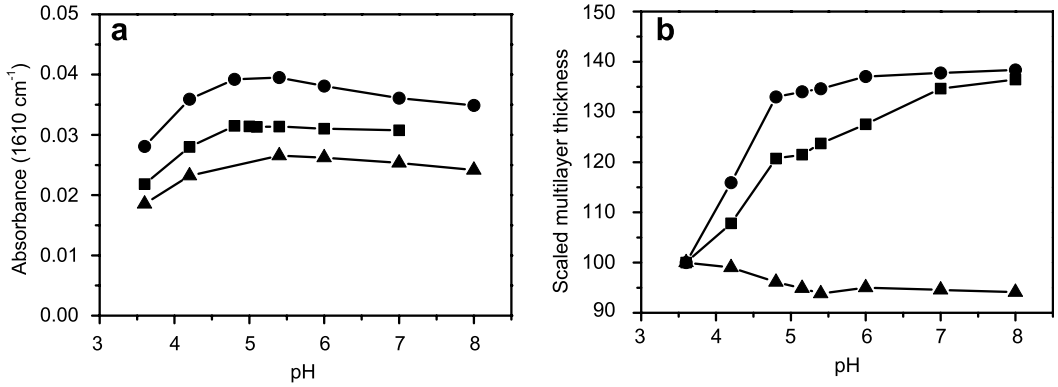

Fig. 8. Infrared carboxylate peak absorbance (1610 cm^{-1}) versus pH (a) and scaled multilayer thickness versus pH (b) for the titration of pectin/PLL/protein multilayers for proteins: BSA (●); BLG (▲); and gelatin (■).

disassembled at pHs close to the proteins IEP, the pectin/PLL/protein multilayers all remained intact.

4. Discussion

In solution, polyelectrolytes can form complexes and coacervates with proteins. Complex formation can occur when both polyelectrolyte and protein carry charge of the same sign, while coacervation generally occurs when polyelectrolyte and protein carry charge of opposite sign. Recent research on BLG/pectin solutions has shown that both types of structure can form from these mixtures (Girard, Sanchez, Laneuville, Turgeon, & Gauthier, 2004). The adsorption of proteins to the surface of polyelectrolyte multilayers, is dependent on the charge of the uppermost layer. When the protein carries a small net charge of the same sign as the uppermost polyelectrolyte a limited adsorption is observed, consistent with the formation of a surface monolayer (Ladam et al., 2000, 2001; Salloum & Schlenoff, 2004). The origin of the limited adsorption could involve a process similar to protein/polyelectrolyte complexation in solution, where the polyelectrolyte interacts with oppositely charged patches on the protein surface, even though the net charge on the protein is of the same sign as the polyelectrolyte. Alternatively the protein could diffuse through the uppermost layer to interact with the underlying oppositely charged layer (Ladam et al., 2001; Salloum & Schlenoff, 2004). When the uppermost layer carries a charge opposite in sign, a more substantial adsorption is observed (Ladam et al., 2001; Salloum & Schlenoff, 2004) Under these conditions, the oppositely charged polymeric components can be sequentially deposited to form a multilayer which has a high capacity for the protein component (Izumrudov et al., 2005). The pectin/protein multilayers fabricated in this study conform to this type of behaviour.

Comparison of the conditions for pectin/protein complexation and multilayer dissolution indicates the same attractive interactions occur in both systems. The dissolution pH of the pectin/β-lactoglobulin multilayer (Fig. 4c and d) coincides with the limiting pH at which Girard et al. (Girard et al., 2004) found solution state high-methoxyl pectin/β-lactoglobulin complexes (pH 5.12 for 1:5 pectin–protein mixtures). Thus the pectin/protein interactions weaken as the pH is increased, and the system returns to a solution state. A further comparison can be made with the behaviour of oil-in-water emulsions stabilised with β-lactoglobulin/pectin membranes (Guzey, Kim, & McClements, 2004). The same interactions appear to affect the ζ-potential of these droplets, controlling whether the pectin is adsorbed to the surface of the droplets or is primarily in the aqueous continuous phase.

On a mass basis there is substantial deposition of the protein carrying an average net positive charge on a negatively charged pectin layer. The hydration of the pectin layer was estimated by comparing the mass of pectin deposited determined by FTIR-ATR, with the hydrated mass determined by QCMD. The water content of 55–92% w/w shows that the pectin/protein multilayer has a relatively open structure which allows access of the proteins. The pectin chains with their relatively long persistence length (Cros, Garnier, Axelos, Imberty, & Perez, 1996) have the capacity to bind a number of protein molecules. For the pectin/protein layers, disassembly of the structures occurred in the vicinity of the isoelectric point of the protein.

The behaviour of mixed pectin/PLL/protein structures was more complex. PLL and pectin were sequentially deposited to form 4 layers with a pectin layer uppermost. There was substantial binding of the globular protein (BSA/BLG) to the pectin layer, at a solution pH where the protein carried a net positive charge. This deposition of protein allowed the subsequent deposition of a further pectin layer. The subsequent addition of PLL displaced a large fraction of the globular protein. This shows PLL and protein are mobile within our multilayers. Using fluorescence recovery experiments on hyaluronic acid–poly-L-lysine multilayers Picart et al. (2002) have previously been shown they exhibit complex dynamics with the hyaluronic acid immobile and the PLL mobile. Our results also suggest that the PLL has a higher affinity for the pectin compared to the globular protein. A similar phenomenon has recently been reported whereby PLL was displaced from a hyaluronic–poly-L-lysine multilayer by the addition of a synthetic polycation, poly(allylamine) (Ball et al., 2005). Under conditions when species are mobile within the multilayers it appears they will exhibit exchange for species with higher binding affinities.

5. Conclusions

Pectin/protein layer-by-layer deposited multilayers can be fabricated at pH 3.6, below the IEP of the proteins, for both globular and flexible proteins. The multilayers are protein rich and, for the globular proteins, the multilayer carries a net positive charge. While the multilayers fabricated from the globular proteins are dense (mass fractions ~40% w/w), those fabricated from gelatin are relatively hydrated (~16% w/w). Titrating the multilayers to pHs above the IEP of the globular proteins resulted in disassembly, confirming the critical role of electrostatic interactions in these structures.

While proteins can be deposited onto pectin/PLL multilayers further deposition of PLL can result in displacement of the globular proteins from the multilayers. FTIR-ATR reveals increased pectin carboxyl ionization as the PLL displaces the globular protein and shrinks the multilayer. These mixed multilayers are stable to increases in pH above the IEP of the proteins.

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