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Enzymatic degradation of poly-L-lysine-polygalacturonic acid multilayers

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ABSTRACT

The action of polygalacturonase on layer-by-layer deposited multilayer films composed of poly-L-lysine and polygalacturonic acid was examined using a quartz crystal microbalance with dissipation monitoring (QCMD), dual polarisation interferometry (DPI) and atomic force microscopy. These techniques were used to determine changes in polymer mass, density, thickness, morphology and hydration of the multilayers in response to enzymatic degradation.

The degradation of the multilayer films by polygalacturonase was studied in the range 0.01–1.0 activity units mL^{-1} ($U\,mL^{-1}$). The QCMD and DPI results show that addition of polygalacturonase solution with an activity of $1\,U\,mL^{-1}$ led to an almost complete disintegration (over 80% mass loss) of the PLL–PGA structure within 20 min, whilst a polygalacturonase solution with the lowest activity had negligible effect on PLL–PGA films. It was found that the multilayer film with PLL uppermost slows the degradation, an effect which persisted throughout the course of the degradation.

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1. Introduction

Polyelectrolyte multilayers (PEMs) were introduced in the early 1990s (Decher, Hong, & Schmitt, 1992; Decher, 1997; Lvov, Decher, & Sukhorukov, 1993) as structures consisting of oppositely charged macromolecules assembled through electrostatic interactions. In PEMs the attraction between the polyanions and polycations are employed in the layer-by-layer deposition process to structure a film with strong interactions between the successive layers. The extensive studies on PEMs carried out over the past two decades has revealed that a great variety of materials can be incorporated into multilayer films and new functional structures can be formed (Dubas & Schlenoff, 2001; Hoogeveen, Stuart, Fleer, & Bohmer, 1996; Krzeminski et al., 2006; Ogawa, Ogawa, & Kokufuta, 2004; Picart et al., 2001; Schneider et al., 2007). Moreover, manipulation of the assembly conditions has led to the fabrication of polyelectrolyte multilayer films of a desired thickness, surface morphology and internal composition. By controlling pH, Shiratori and Rubner (2000) showed that it was possible to change the thickness of an adsorbed polycation or polyanion layer from 0.5 to 8 nm. Salt and pH dependent multilayer growth has also been reported for weak anionic polysaccharides, such as alginate/poly-L-lysine (Elbert, Herbert, & Hubbell, 1999), hyaluronan/poly-L-lysine (Burke & Barrett, 2003), and hyaluronan/chitosan (Richert et al., 2004b). Additionally, Glinel et al. (2007) had shown that varying charge densities of the multilayer components affect the intermolecular relations within the constructed films causing either collapse or swelling of the multilayer structure.

The great potential of the layer-by-layer approach to create new functional structures has led to a variety of applications of the polyelectrolyte multilayer films in areas such as biomedical devices, surface modification, biosensors, dye, and photoreaction inhibitors (Boudou, Crouzier, Ren, Blin, & Picart, 2010; Decher & Schlenoff, 2003; Elsabee, Abdou, Nagy, & Eweis, 2008; Prevot, Dejugnat, Mohwald, & Sukhorukov, 2006). Recently, there has been great interest in using PEMs as a method to encapsulate and site-deliver nutrients in a controlled manner along the gastrointestinal tract (Maltais, Remondetto, & Subirade, 2009). This application, however, requires not only the knowledge of how the multilayer is formed, but also an understanding of how the stability of multilayer films are then affected by various environmental stresses, such as pH, salt, temperature and enzymes. As previously shown by Möhwald et al. (Sukhorukov, Antipov, Voigt, Donath, & Mohwald, 2001), the permeation of chemicals through the shell of multilayer capsules can be tuned by changes in pH. Rubner et al. (Mendelsohn et al., 2000) and Moffat, Noel, Parker, Wellner, and Ring (2007) have also reported pH-induced changes in the stability and morphology of multilayer films formed on a flat substrate. Dubas and Schlenoff (2001) reported swelling behaviour leading to the disassembly of a polyelectrolyte network consisting of one strong and one weak polyelectrolyte at a salt concentration greater than 0.6 M NaCl.

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Although there is considerable literature on pH- and salt-responsiveness of multilayer films, there is less data concerning enzymatic degradation of the multilayer films available. Ren, Ji, and Shen (2006) stressed the importance of the enzymatic biodegradability of films for gene delivery and gene therapy, and showed that PLL–DNA multilayer films could be used potentially for this application. Schaaf et al. (Etienne et al., 2005) presented *in vitro* and *in vivo* studies on the degradability of chitosan/hyaluronan PEMs by lysozyme and amylase. They reported that films crossed-linked with water-soluble carbodiimide in combination with *N*-hydroxysulfosuccinimide were more resistant to enzymatic degradation as compared to native chitosan–hyaluronan PEMs. In addition, Akashi et al. (Serizawa, Yamaguchi, & Akashi, 2002) reported that, for biomedical applications, one of the most important requirements is the biodegradability of ultrathin polymer films coated on material surfaces, and thus investigated enzymatic degradation of chitosan/dextran sulphate multilayers. The study conducted by Akashi et al. (Serizawa et al., 2002) demonstrated that chitosan–dextran sulphate multilayers with dextran sulphate as a top layer were hydrolysed with chitosanase more quickly, when compared to the same film having chitosan as the top layer, even though the chitosanase hydrolyses chitosan. It has been established that this unique hydrolysis resulted from electrostatic condensation of the chitosanase onto the top of the dextran sulphate layer, which subsequently led to hydrolysis of chitosan within the multilayer. Recently, Sasaki, Noel, and Ring (2008) used the quartz crystal microbalance with dissipation (QCMD) technique to monitor the α-amylase hydrolysis of potato amylopectin and reported the protective effect of a chitosan layer on the α-amylolysis of potato amylopectin. These different outcomes required further investigation as to how the multilayer structure affects the hydrolysis of the affected constituent of the film.

In this current study, we have investigated the integrity of PLL–PGA multilayer films in response to the action of polygalacturonase. Poly-L-lysine is a biocompatible and non-toxic weak polyelectrolyte that has been used for biomedical applications, as a component of composites which support primary cell growth, biosensors and capsules for drug release (Richert et al., 2004a; Tryoen-Toth et al., 2002). It has also proven to be a suitable natural preservative for food products such as boiled rice, vegetables, soups and fresh fish for sushi (Hiraki et al., 2003). Polygalacturonic acid (PGA) in a partially esterified form is the main component of pectin. Pectin is a polysaccharide extract from the cell walls of edible plants. The extract contains linear polymer chains of α(1 → 4)-D-galacturonosyl units with small quantities of neutral sugars present as short sidechains in structures known as rhamnogalacturonan regions. In pectin extracts, the PGA is partially esterified with methyl groups, and blocks of free carboxylate groups enable the pectin to form cooperative electrostatic interactions with polycations. PGA is formed by a partial hydrolysis of side groups of the pectin chains. During the process of ripening, pectins are broken down in the hydrolysis of the glycosidic bonds by a group of enzymes recognised as pectinase, which are present in raw fruits (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999; Daas, Voragen, & Schols, 2000; Schols, Posthumus, & Voragen, 1990).

In recent studies (Krzeminski et al., 2006) we have reported continuous multilayer growth of PLL–pectin films performed at 20 °C. PLL–pectin multilayer growth followed an exponential regime, indicating that PLL interpenetrated the structure during assembly (Krzeminski et al., 2006). The interactions between the layers were determined by the degree of esterification (DE) of the pectin, showing that the affinity for PLL increased with decreasing DE of pectin. Thus, adsorption of PLL and PGA occurred rapidly, showing strong interactions between the consecutive layers. Due to the increasing interest in studies of PEMs stability to various stresses that are crucial for a number of applications (Boudou et al., 2010; Decher & Schlenoff, 2003), Moffat et al. (2007) studied the stability of PLL–pectin multilayer films to environmental stresses, such as salt and pH, and reported the partial disassembly of PLL–pectin multilayer films under acidic pH conditions. This study reports an extension of such work to determine PEM stability to enzymatic degradation. The PLL–pectin multilayers were assembled at 37 °C and the rate of the enzymatic hydrolysis of polygalacturonic acid embedded in PLL–PGA multilayer films was investigated using QCMD, dual polarisation interferometry (DPI), and atomic force microscopy (AFM). In this study a polygalacturonase preparation from the fungus *Aspergillus niger* was used. This contains a mixture of highly homologous endo-acting enzymes capable of hydrolysing polygalacturonic acid. Structures have been solved for the best characterised PGases, i.e., enopolygalacturonase I and II, which shows both are folded into compact right-handed β-helical structures containing 10 complete turns with overall dimensions 6.5 nm × 3.5 nm × 3.5 nm and a molecular weight of 35 kDa (van Pouderoyen, Snijder, Benen, & Dijkstra, 2003; van Santen et al., 1999). A recent study on α-amylase hydrolysis of starch polysaccharides immobilised on a QCMD sensor (Sasaki et al., 2008) proved that QCMD is a straightforward tool for the *in vitro* analysis of multilayer growth and enzymatic digestion of the PEMs. The QCMD technique monitors changes in the resonant frequency of the QCMD sensor due to adsorption or deposition of the polyelectrolyte material onto its 'active' surface. Therefore, we employed the QCMD technique to monitor PLL–pectin multilayer formation followed by the enzymatic degradation. In order to provide further information regarding the changes in polymer mass, thickness, density and morphology of the films in response to polygalacturonase action, we have acquired additional data using DPI and AFM measurements. We have also extended the study to account for the protective effect of the top PLL layer on the rate of enzymatic degradation of PLL–pectin multilayers and established that it slows down the rate of hydrolysis.

2. Materials and methods

2.1. Polyelectrolyte and enzyme solutions

Poly-L-lysine hydrobromide with a mean degree of polymerisation of 70 (PLL) was obtained from Sigma. It was previously shown to have a hydrodynamics radius of 2.6 nm. Polygalacturonic acid (PGA, M_W = 25–50 kDa, hydrodynamic radius, 7.1 nm) (Krzeminski et al., 2006) was supplied by Fluka, Biochemika. PGA has been analysed in detail by Ryden, MacDougall, Tibbits, and Ring (2000). Solutions of 0.6 mg mL^{-1} were used for the multilayer assembly and prepared by dissolving the biopolymer in 10 mM pH 7.0 phosphate buffer, containing 30 mM NaCl. The pH was adjusted by adding 0.1 M NaOH. An aqueous glycerol solution of polygalacturonase (EC 3.2.1.15) from *A. niger* (16 U mg^{-1} polygalacturonase, 46 mg mL^{-1} (Lowry)) were obtained from Sigma. The polygalacturonase solutions of different concentrations ranging from 0.01 to 1 U mL^{-1} were prepared at pH 4.0 in 50 mM sodium acetate buffer supplied by BDH Laboratory Supplies.

2.2. Multilayer formation and enzymatic degradation

The multilayer structures were prepared on silicon dioxide substrate by electrostatic layer-by-layer assembly (Decher, 1997) at 37 °C, following the procedure described previously (Krzeminski et al., 2006). Prior to deposition the substrate surfaces were cleaned using a UV–ozone chamber (Bioforce Nanosciences, Inc., IA, USA), then rinsed thoroughly with water and dried with N_2. The PLL–PGA multilayer film was prepared through sequential injections of PLL and PGA solutions 0.5 mL (QCMD) and 0.1 mL (DPI). Each depo-

sition step lasted 4 min. Between each polyelectrolyte deposition step the substrate with the polymer film was rinsed with buffer to remove any excess of free polyelectrolyte. This procedure was repeated until 10 or 11 consecutive layers were formed.

The multilayer films were then treated with 0.5 mL (QCMD) and 0.3 mL (DPI) of polygalacturonase solutions prepared as described above. A range of polygalacturonase activities 0.01, 0.1, 1 U mL^{-1} were used in this study. In addition, the effect of sodium acetate buffer, without polygalacturonase, on the formed PEMs was tested.

2.3. Quartz crystal microbalance with dissipation monitoring (QCMD)

Measurements were carried out using a D300 QCMD (Q-Sense AB, Västra Frölunda, Sweden) with a QAFC 302 axial flow measurement chamber. The PGA-PLL multilayers were deposited on SiO_2-coated surface of a disc-shaped AT-cut piezoelectric quartz crystal positioned between two electrodes. The exposed area of quartz crystal has a diameter of about 11 mm giving a surface area of about 0.95 cm^2 which, with a cell depth of about 0.84 mm, gives a cell volume of 80 μL. In the QCMD instrument the crystal disc oscillates at a resonant frequency of approximately 5 MHz. The detected increased frequency shifts ($\Delta f_n/n$) are related to a deposition of mass (Δm) onto the surface of substrate and mass loss was indicated by a decrease of $\Delta f_n/n$. When the mass is deposited evenly and the formed film is rigid the change of frequency is related to mass adsorption through the Sauerbrey equation (Sauerbrey, 1959):

$$\Delta m = \frac{-C\Delta f_n}{n} \quad (1)$$

where C is the mass sensitivity constant for a 5 MHz crystal, $C = 17.7$ ng/cm^2 Hz and n is overtone number, $n = 3, 5, 7$. However, for viscoelastic materials, the Sauerbrey relation leads to an underestimate of the deposited hydrated mass. In order to extract information regarding the viscoelastic nature of the multilayer, the changes in dissipation (ΔD_n) were recorded during multilayer assembly: the more viscous the film the higher the measured ΔD_n value.

The QCMD response to the formation of dissipative viscoelastic multilayer films was then modelled with the Voigt model using Q-Tools software supplied by Q-sense AB. Q-Tools uses a simplex algorithm to find the minimum of the sum of the squares of the scaled errors between the model output and the experimental data. The software requires assumed values for density, ρ_2 and viscosity, η_2 of the bulk liquid, and a fixed density of the film, ρ_1: estimated to be equal to 1000 (kg/m^3) and 0.69 (mPas), and 1100 (kg/m^3), respectively.

After each measurement the silica substrate and microbalance chamber were rinsed thoroughly with deionised water and a 2% solution of Hellmanex II alkaline surfactant (Hellma GmbH & Co, UK), then flushed with water, and dried using compressed nitrogen. The silica chip was then treated in an ozone–UV chamber for 10–15 min to remove any organic residue from the active surface, rinsed with water and dried with nitrogen.

2.4. Dual polarisation interferometry (DPI)

The measurements were carried out using an AnaLight Bio200 DPI (Farfield Scientific, Crewe, UK) with a silicon oxynitride sensor chip (Halthur, Claesson, & Elofsson, 2006; Lu, Swann, Peel, & Freeman, 2004). The chip consists of a sandwich of two horizontally stacked waveguides, a sensing waveguide on top of a reference waveguide, separated by an opaque cladding material. A gasket on top of the sensing waveguide forms two measurement chambers, each 1 mm wide, 17 mm long and 1 mm deep (17 μL volume).

DPI is a noninvasive optical technique that is used to determine the effective refractive index (RI) and thickness of a thin film based on the interaction of the adsorbed material on the evanescent field of polarised light transmitted through the waveguides by total internal reflection. Laser light illuminating one end of the chip is switched between two polarisation modes: the light travels along the waveguides and on exiting the chip the interaction with the deposited film produces a phase shift in the interference fringe pattern, which is detected by a camera located in the far field. Each state of polarisation will produce a different interference fringe patterns. By solving Maxwell's equations simultaneously for the two modes of polarisation unique values can be obtained for the refractive index and thickness of the adsorbed film.

At the start of each experiment the sensing chip was calibrated with 40% w/w ethanol and water. Prior to the experiment the chips were treated with the UV–ozone for 20 min. Multilayers were assembled by injecting 100 μL of biopolymer solution into a continuously flowing buffer at a flow rate of 20 μL min^{-1} using an HPLC sampling valve. After each injection there was a rinsing step of 5 min duration. The de Feijter formula (Feijter, Benjamins, & Veer, 1978) was used to determine the adsorbed mass per unit area Γ (ng cm^{-2}) from the mean optical adsorbed film thickness, d_f, the refractive index of the film, n_f and dn/dc, the refractive index increment of the deposited species.

$$\Gamma = d_f \frac{n_f - n_{\text{buffer}}}{dn/dc} \quad (2)$$

2.5. Atomic force microscopy (AFM)

AFM topographic images were obtained in liquid using an MFP-3D BIO instrument (Asylum Instruments, Santa Barbara, CA). Images were recorded in tapping mode with Olympus AC40TS 'BioLever Mini' cantilevers with a nominal spring constant of 0.1 N m^{-1}. The cantilevers were operated at just below their second Eigen frequency. In order to image samples prepared under the same conditions as those used during the QCMD measurements, the assembly of the polyelectrolyte multilayers, followed by the enzymatic degradation, were performed on mica wafers within the QCMD flow chamber. The samples were transferred under liquid to the AFM. For imaging the mica discs were attached to PTFE coated glass slides (Electron Microscopy Sciences, Hatfield, PA) and imaged under liquid.

3. Results and discussion

3.1. Multilayer deposition

In this study the multilayers were first deposited onto silicon oxide substrates and subsequently treated with enzyme solutions. The multilayer assembly of all 10 and 11-layer PLL–PGA films were performed at pH 7.0 and 37 °C, and the adsorption of the PLL–PGA hydrated mass was monitored in real time using the QCMD and DPI. The simultaneous measurements of the QCMD's quartz crystal oscillator frequency shifts ($\Delta f_n/n$) and the dissipation (ΔD_n) were recorded at the three overtones ($n = 3, 5, 7$) and the recorded changes displayed as a function of time are shown in Fig. 1.

Adsorption of PLL and PGA occurred rapidly with a plateau being reached after approximately 2 min (Fig. 1). The PLL adsorption was indicated by a rapid decrease in $\Delta f_n/n$ with a minimal increase in ΔD_n. In contrast during the PGA deposition the decrease in $\Delta f_n/n$ was accompanied by a significant increase in ΔD_n. The PLL–PGA multilayer assembly process was almost irreversible, with only minor amounts of non-absorbed polyelectrolyte being removed

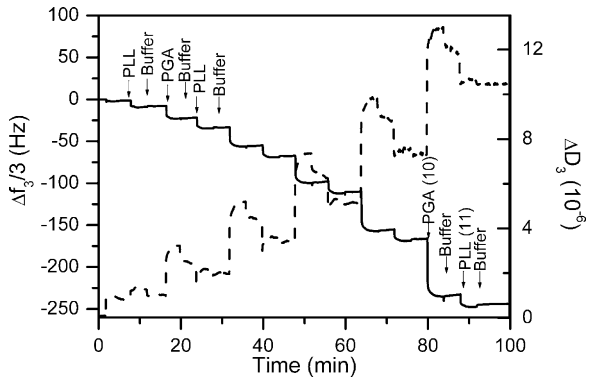

Fig. 1. Evolution of $\Delta f_3/3$ (—) and ΔD_3 (- - - -) during the assembly of 10 and 11-layer PLL–PGA films at 37 °C.

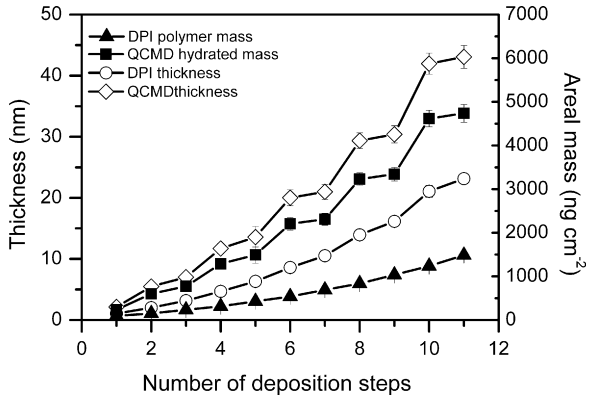

Fig. 2. Evolution of DPI polymer mass (–▲–), QCMD hydrated mass (–■–), DPI thickness (–○–) and QCMD thickness (–◇–) during the assembly of 10 and 11- layer PLL–PGA films at 37 °C.

upon rinsing with the buffer solution following each adsorption of a polymer layer. After each PLL deposition an increase in PGA adsorption was observed. This observation indicated that during the multilayer assembly, PLL interpenetrated the structure, and resulted in a non-linear multilayer growth regime (Porcel et al., 2006).

The hydrated mass adsorbed, including biopolymer, water, and salt coupled to the biopolymer layer was estimated on the basis of the value of $\Delta f_n/n$, and the film thickness was estimated assuming uniform film formation with a density of 1100 kg m^{-3}. Due to the significant changes in dissipation observed during the PLL–PGA multilayer assembly, which indicated the formation of a viscoelastic film, the hydrated mass of 10 and 11 layer PLL–PGA films were calculated based on the Voigt model (Voinova, Rodahl, Jonson, & Kasemo, 1999). The total frequency change of a 10-layer assembly was observed to be -220 ± 20 Hz, which corresponded to the deposition of hydrated mass of 4600 ± 500 ng cm^{-2} (Fig. 2). The $\Delta f_n/n$ of a 11-layer assembly was observed to be slightly higher, with the total observed $\Delta f_n/n$ being -240 ± 10 Hz, which corresponded to a total deposition of 4800 ± 500 ng cm^{-2} of hydrated mass (Fig. 2).

Whilst the QCMD technique provided information about the hydration of the PLL–PGA multilayer films by monitoring changes in $\Delta f_n/n$, and ΔD_n the DPI data provided information regarding the thickness and RI, and consequently the polymer mass adsorbed during the assembly based on measurements using T_E (transverse electric) and T_M (transverse magnetic) modes (Fig. 3).

The thickness and RI changes that occurred during the assembly were estimated under the assumption that the formed PLL–PGA films were homogenous. The Feijters equation (Feijter et al., 1978)

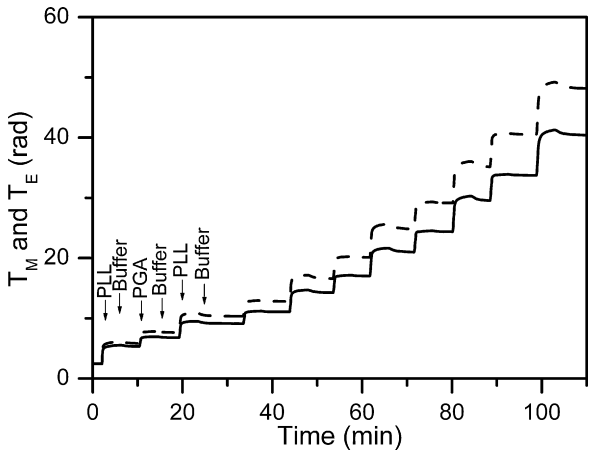

Fig. 3. Evolution of the transverse magnetic (T_M (—)) and transverse electric (T_E (- - -)) during the assembly of 10 and 11-layer PLL–PGA films at 37 °C.

was used to estimate the polymer mass adsorbed based on the buffer solution RI being 1.3336 and dn/dc having a value of 0.15 mL g^{-1} for the PLL–polysaccharide multilayer film mixture (Halthur & Elofsson, 2004).

The evolution of thickness and mass during the assembly of PLL–PGA films is shown in Fig. 2. We found that the PLL–PGA multilayer films were relatively dense (0.5 g cm^{-3}) and the 10- and 11-layer film thicknesses were 21.1 ± 1 nm and 23.1 ± 1 nm, respectively. The evolution of polymer mass during growth of the PLL–PGA multilayers (Fig. 2) followed a linear growth regime during the formation of the first 4 layers, with the subsequent layers showing a non-linear growth regime, leading to a total polymer mass deposition of 1200 ± 50 ng cm^{-2} for 10-layer films and 1500 ± 70 ng cm^{-2} for 11-layer films. Modifications to the cleaning procedure of DPI chips prior to the adsorption experiments led to an increase in total PLL–PGA mass adsorption compared with previously reported values (Westwood, Noel & Parker, 2010, in press). The estimated water content, based on QCMD and DPI measurements, showed a high degree of hydration of approximately 75% and 70% for 10-layer and 11-layer PLL–PGA films, respectively. The difference in hydration between 10 and 11-layer PLL–PGA films is in agreement with the conclusions of Notley, Eriksson, and Wagberg (2005), who reported that the properties of the multilayer films were highly dependent on the polylelectrolyte present in the topmost layer. In this study we observed a higher dissipation for 10-layer PLL–PGA (12×10^{-6}) compared to an 11-layer PLL–PGA film (10.5×10^{-6}) indicating that the 10-layer film was more viscous before addition of the extra layer of PLL.

The findings presented here reveal that the adsorbed mass and growth characteristic of 10-layer PLL–PGA assembled at 37 °C are the same as those of 10-layer PLL–PGA multilayers assembled at 20 °C, as previously reported (Westwood et al., 2010, in press). Although, it has been established that the formation of PEMs at higher temperatures yield thicker films, due to changes in the polymer–solution interactions and the higher internal mobility at which the polyelectrolytes can swell into different conformations that are unfavourable at lower temperatures (Buscher, Graf, Ahrens, & Helm, 2002; Halthur, Claesson, & Elofsson, 2004), this study does not support this conclusion. We have shown that PLL–PGA multilayer growth was temperature independent, and pairing PLL (pK_a of 9.0) (Girod et al., 2004) with PGA (pK_a of 3.5) (de Kerchove & Elimelech, 2007), which at the pH of 7.0 were fully charged, resulted in a strong electrostatic interaction between consecutive layers regardless the temperature at which the films were assembled.

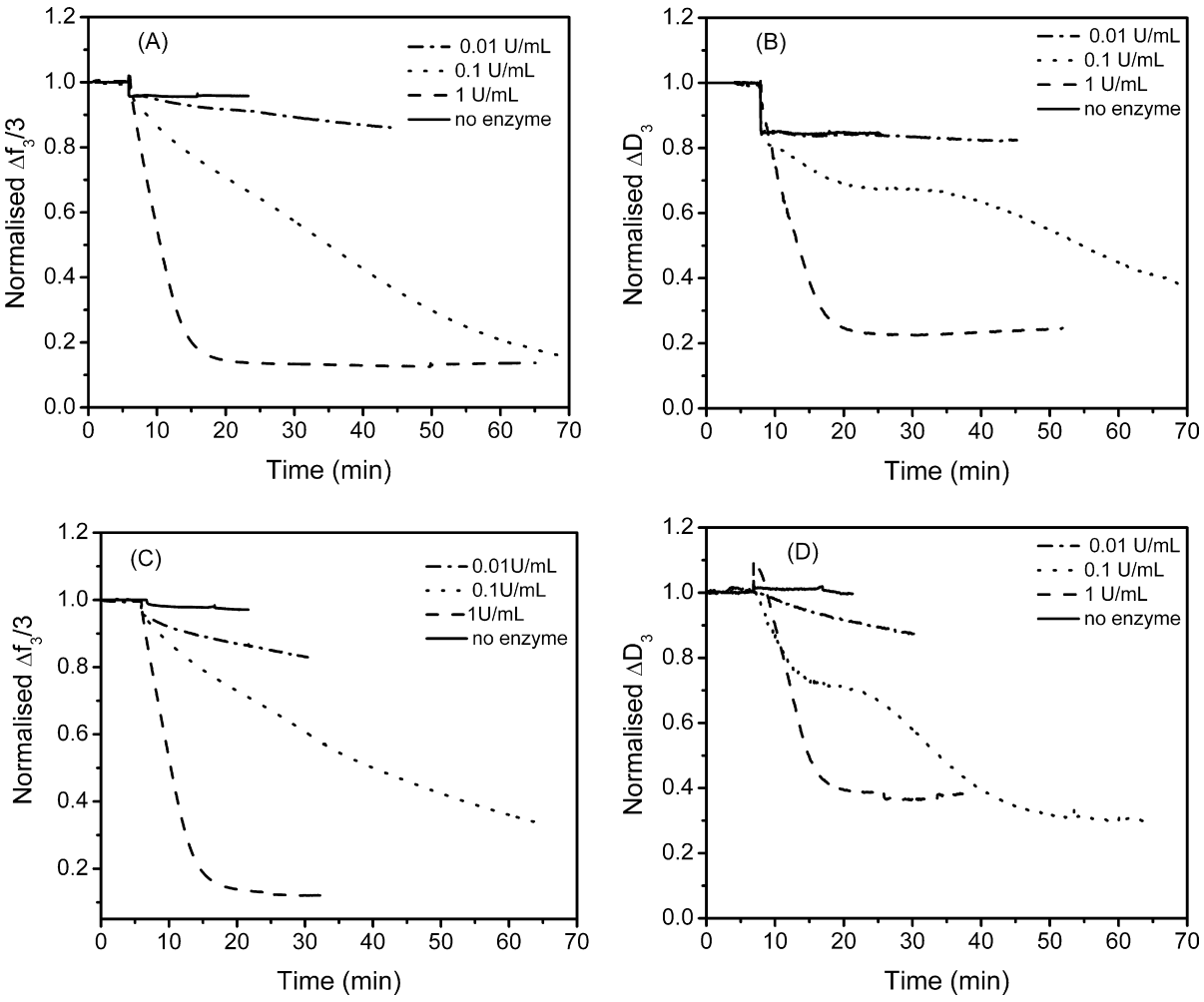

Fig. 4. Concentration dependent effect of polygalacturonase on 10 and 11-layer PLL–PGA films measured with QCMD as a function of time at 37 °C. Effect of polygalacturonase on (A) normalised $\Delta f_3/3$, and (B) normalised ΔD_3 measured for 10-layer PLL–PGA film 10-layer. Effect of polygalacturonase on (C) normalised $\Delta f_3/3$, and (D) normalised ΔD_3 measured for 11-layer PLL–PGA films. The concentration range of polygalacturonase solutions used in this study ranged between 1 and 0.01 U mL^{-1}.

3.2. Enzymatic degradation

3.2.1. QCMD

After the assembly of the 10 and 11-layer PLL–PGA films were complete they were subjected to the action of an enzyme (polygalacturonase) solution, and the effects of the enzymatic hydrolysis of the polygalacturonic acid embedded in the multilayers was monitored in the real time using QCMD, DPI and AFM. The enzymatic degradation of PLL–PGA films was examined at 37 °C, at three different enzyme concentrations corresponding to activities: 0.01, 0.1 and 1 U mL^{-1}. Polygalacturonase was dissolved in a 50 mM sodium acetate buffer with pH 4.0, the pH at which it exhibits optimal activity (Dinu, Nechifor, Stoian, Costache, & Dinischiotu, 2007; Mill & Tuttobello, 1961).

Fig. 4 shows the frequency changes observed during the enzymatic degradation of a 10-layer PLL–PGA film at different enzyme concentrations. The action of 1 U mL^{-1} polygalacturonase solution on the 10-layer PLL–PGA films led to a sharp increase of $\Delta f_n/n$, reaching an approximate constant value of -40 ± 5 Hz in approximately 20 min after injection of the polygalacturonase solution. The increase of $\Delta f_n/n$ measured with the QCMD clearly indicated a significant reduction of hydrated mass. The amounts of immobilised and released hydrated masses from PLL–PGA structure were estimated to be 4600 ± 500 ng cm^{-2} and 3800 ± 100 ng cm^{-2}, respectively. This showed that 83% of the PLL–PGA hydrated mass was released during the hydrolysis within a period of 20 min. Lowering the enzyme concentration to 0.1 U mL^{-1} resulted in a slower degradation of the 10-layer PLL–PGA films compared to the degradation at 1 U mL^{-1} polygalacturonase. The $\Delta f_n/n$ values gradually increased with time and, after approximately 70 min from the injection of the polygalacturonase solution, reached the same constant value of -40 ± 5 Hz, as observed during the exposure of a 10-layer PLL–PGA film to a 1 U mL^{-1} polygalacturonase solution.

At the initial stage of the hydrolysis process, as shown in Fig. 4A, the rate of frequency change was linear. This allowed us to estimate and compare the initial hydrolysis rates obtained at the different concentrations used in this study. The initial 10-layer PLL–PGA film hydrolysis rate in response to 1 U mL^{-1} polygalacturonase was 0.11 min^{-1}, and that in response to 0.1 U mL^{-1} polygalacturonase was 0.0144 min^{-1}, which demonstrated that the hydrolysis conducted with 1 U mL^{-1} polygalacturonase was 7 times faster when compared to the hydrolysis rate conducted with 0.1 U mL^{-1} polygalacturonase solutions.

Finally, the exposure of the 10-layer PLL–PGA film to a 0.01 U mL^{-1} polygalacturonase solution led to a $\Delta f_n/n$ increase of only 20 ± 5 Hz in approximately 70 min, which corresponded to a reduction of only 10% of the total PLL–PGA hydrated mass, leading to the conclusion that 0.01 U mL^{-1} of polygalacturonase had a minor effect on 10-layer PLL–PGA films over this experimental timescale. As the PLL–PGA multilayer growth was performed at pH

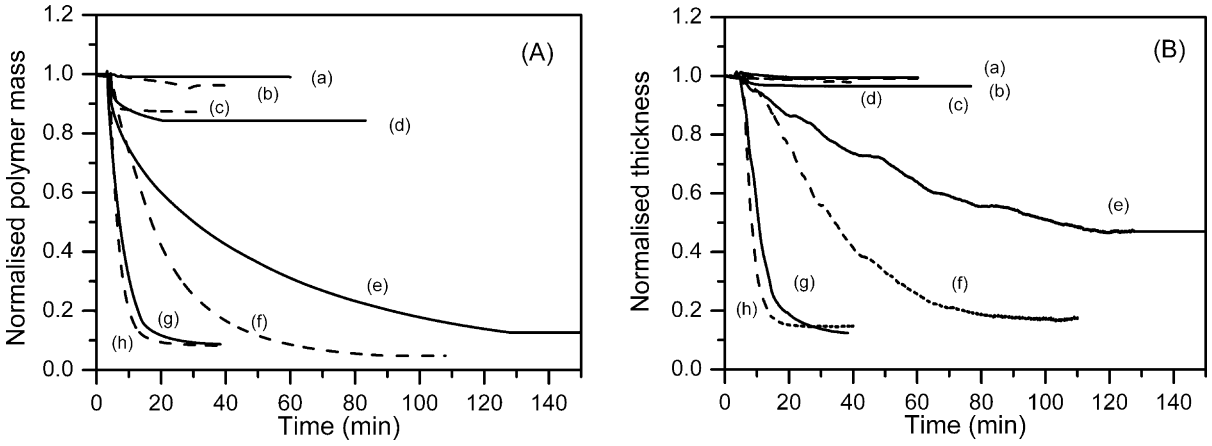

Fig. 5. Concentration dependent effect of polygalacturonase on 10 (- - - -) and 11-layer (—) PLL–PGA films measured with DPI as a function of time at 37 °C. Effect of (b) no enzyme, (c) 0.01 U mL^{-1}, (f) 0.1 U mL^{-1}, and (h) 1 U mL^{-1} of polygalacturonase on (A) the normalised polymer mass, (B) normalised thickness measured for 10-layer PLL–PGA films. Effect of (a) no enzyme, (d) 0.01 U mL^{-1}, (e) 0.1 U mL^{-1}, and (g) 1 U mL^{-1} of polygalacturonase on (A) the normalised polymer mass, (B) normalised thickness measured for 11-layer PLL–PGA films.

7.0, and the polygalacturonase was prepared in a sodium acetate solution of pH 4.0, the effect of buffer pH on the PLL–PGA films was analysed. Injection of the pH 4.0 sodium acetate solution caused an increase in frequency by 10 Hz, which indicated the stability of the PLL–PGA films to a pH change from pH 7.0 to 4.0. The stability of PLL–pectin multilayers to changes in pH has been investigated by Moffat et al. (2007) and the results revealed that PLL–pectin films shrunk significantly (30% of the original thickness) in response to a pH change from 7.0 to 4.0. However, the pectin used in that study was highly esterified (degree of esterification, 70.6%), which determined the charge density of pectin. Because the pectin used in that particular study was much less charged when compared to the PGA sample used in this present study, this led to weaker interactions between the PLL and pectin. Thus, the PLL–pectin films were pH responsive within a pH range of 7–4, whereas the PLL–PGA films assembled in this study appear to be stable within the same pH range.

The effect of having the polycation as the topmost layer on the rate of enzymatic degradation of PLL–PGA films in response to polygalacturonase action was also investigated. It was found that 11-layer films immediately underwent hydrolysis when 1 U mL^{-1} polygalacturonase solution was added, revealing only a small change in hydrolysis rate (0.1 min^{-1}) when compared to the degradation of a 10-layer PLL–PGA film (Fig. 4B). As expected a decrease of the polygalacturonase activity to 0.1 U mL^{-1} resulted in a lower degradation rate of 0.016 min^{-1} when compared to the effect of a 1 U mL^{-1} polygalacturonase on 11-layer PLL–PGA films. Although initially the degradation rate was slightly higher for 10-layer PLL–PGA film, after approximately 5 min from the injection of 0.1 U mL^{-1} polygalacturonase, a decrease to 0.014 min^{-1} in the rate of degradation of the 11-layer PLL–PGA film was observed. After 70 min, the loss of hydrated mass reached a value of 65% of the total hydrated mass, clearly indicating a protective effect of the top PLL layer against hydrolysis. Due to the enzyme action the PGA chains are ultimately shortened to oligomers that are then gradually released (desorbed) from the residual films (Daas et al., 1999; Mill & Tuttobello, 1961). Although the initial rate of degradation is comparable to the decomposition of a 10-layer PLL–PGA it is believed that, at the later stage of degradation when a significant amount of PGA has been desorbed from the films, the amount of PLL that remained had collapsed blocking the penetration of the polygalacturonase into the remaining structure and thus slowing degradation at the later stage of degradation. The comparison of the initial degradation rates indicated that the deposition of a PLL layer as the outermost layer slowed the hydrolysis by a factor of 1.1 for 1 U mL^{-1} and 1.2 for 0.1 U mL^{-1} for polygalacturonase solutions, respectively.

3.2.2. DPI

A further, more detailed, investigation of the effect of polygalacturonase activity on PLL–PGA films was carried out using DPI with an emphasis on an analysis of the changes in polymer mass, RI, density and thickness of 10 and 11-layer PLL–PGA films. In order to compare the degradation of the 10 and 11-layer films in response to polygalacturonase solutions of different concentrations, the thicknesses and polymer masses shown in Fig. 5 were normalised using the initial values of optical thickness and polymer mass. This direct comparison of degradation rates revealed that the DPI findings were consistent with the results obtained using the QCMD technique. As with QCMD measurements, DPI revealed that

Table 1
Characteristics of 10 and 11-layer PLL–PGA films before and after enzymatic degradation with polygalacturonase. Averaged over at least four replicates.

	RI	Density (g cm^{-3})	Polymer mass (ng cm^{-2})	Thickness (nm)
10 layers				
Assembly	1.421 ± 0.005	0.582 ± 0.032	1230 ± 50	21.2 ± 1.1
No enzyme	1.427 ± 0.004	0.623 ± 0.023	1220 ± 30	19.5 ± 1.2
0.01 U	1.417 ± 0.005	0.554 ± 0.032	1120 ± 100	20.2 ± 1.1
0.1 U	1.423 ± 0.022	0.600 ± 0.150	130 ± 50	1.4 ± 1.3
1 U	1.463 ± 0.027	0.860 ± 0.181	170 ± 50	2.2 ± 1.0
11 layers				
Assembly	1.428 ± 0.004	0.647 ± 0.056	1470 ± 80	23.2 ± 0.7
No enzyme	1.430 ± 0.001	0.640 ± 0.009	1400 ± 20	21.9 ± 0.7
0.01 U	1.415 ± 0.001	0.545 ± 0.008	1180 ± 30	21.6 ± 0.7
0.1 U	1.391 ± 0.012	0.385 ± 0.069	240 ± 30	6.3 ± 1.4
1 U	1.427 ± 0.005	0.622 ± 0.032	230 ± 40	3.7 ± 0.8

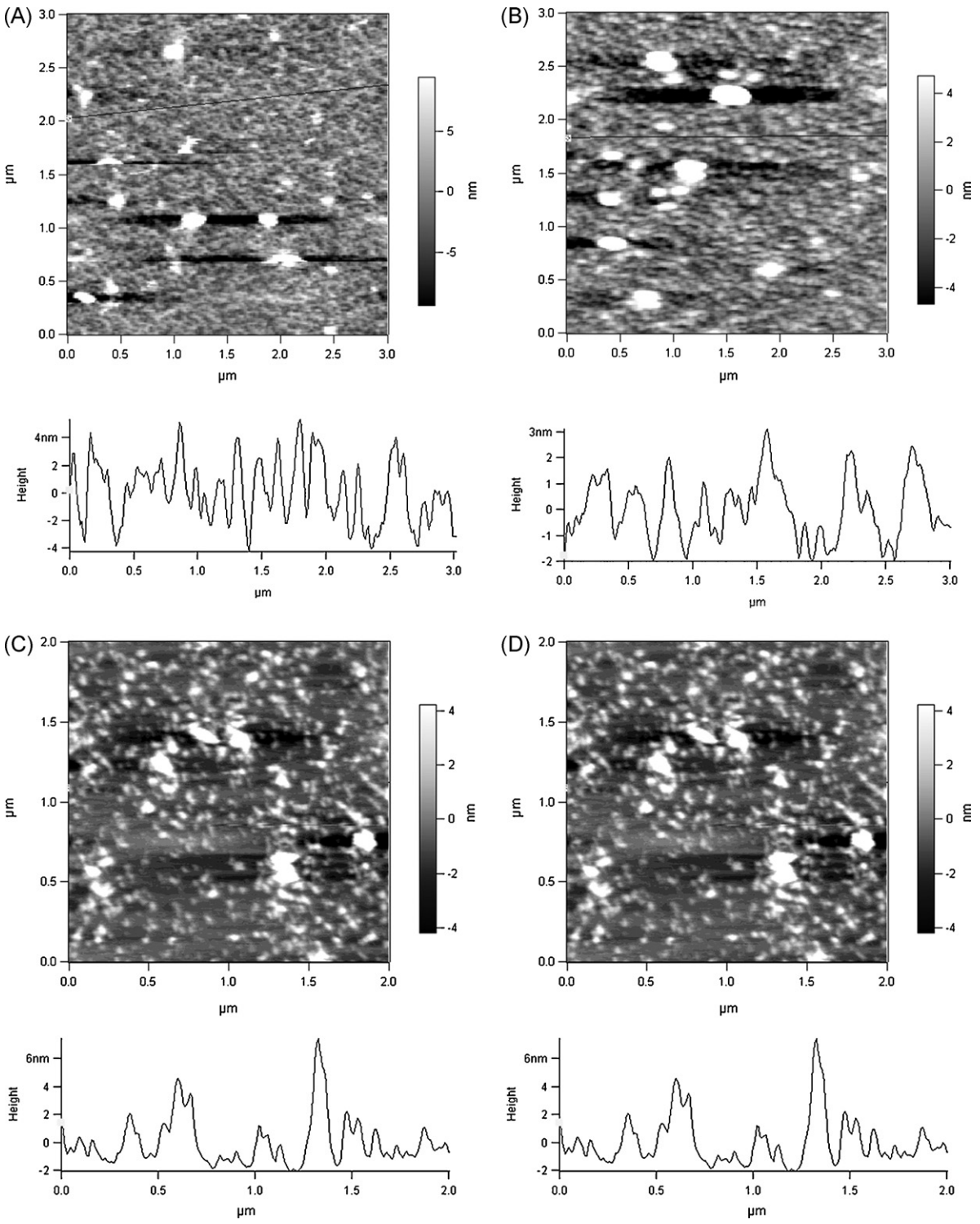

Fig. 6. AFM images of PLL–PGA films. 10-layer PLL–PGA film imaged (A) before, and (C) after the action of polygalacturonase. 11-layer PLL–PGA film (B) before, and (D) after the action of polygalacturonase.

the rate of multilayer degradation by polygalacturonase increased with the enzyme concentration. A fast enzymatic degradation of both 10 and 11-layer PLL–PGA films occurred at the highest activity studied ($1\,U\,mL^{-1}$), with the initial polymer mass loss rates being $0.133\,min^{-1}$, and $0.111\,min^{-1}$, respectively (Fig. 5).

Monitoring changes in the PLL–PGA film thickness and RI, which is directly related to the polymer density, revealed that adding $1\,U\,mL^{-1}$ polygalacturonase to a 10-layer film resulted in a significant thickness reduction from 21.2 ± 1.1 nm to a minimum value of 2.2 ± 1.0 nm during the 20 min incubation, whilst the value of RI increased from 1.421 ± 0.005 to 1.463 ± 0.027 (Table 1). Thus during the degradation of the film the density increased from $0.582 \pm 0.032\,g\,cm^{-3}$ to $0.860 \pm 0.181\,g\,cm^{-3}$. This contrasted with the behaviour for the 11-layer multilayer for which the density of the residual multilayer was $0.622\,g\,cm^{-3}$, a slightly lower density than that originally deposited.

The exposure of the films to a 0.1 U mL^{-1} polygalacturonase solution resulted in slower degradation of the films, and initial degradation rates for 10 and 11-layer PLL–PGA films were estimated to be 0.0312 min^{-1} and 0.0144 min^{-1}, respectively. A decrease of RI in response to the 0.1 U mL^{-1} polygalacturonase solution and a reduction of thickness clearly demonstrated that, due to the polygalacturonase action on both 10 and 11-layer films, the gradual mass desorption led to the formation of much thinner and, in contrast with the behaviour at 1.0 U mL^{-1}, less dense films (Table 1). The comparison of the initial degradation rates indicated that the deposition of a PLL layer as the outermost layer slowed the rate of degradation by a factor of 1.5 for 1 U mL^{-1} and 2.2 for 0.1 U mL^{-1} polygalacturonase solutions, respectively. At the two higher enzyme concentrations the exposure to polygalacturonase solutions resulted in a loss of approximately 87 ± 2% of the total polymer mass for a 10-layer film and 85% for an 11-layer film. A distinct characteristic of the 11 layer films treated with 0.1 U mL^{-1} was their low final density (Table 1). This is the result of the polymer mass (Fig. 5A) decreasing faster than the thickness (Fig. 5B), and indicates a swelling of the residual film as compared with the initially assembled film. It is interesting to note that the effects of the initial uppermost layer persist even after over 2 h of enzymatic degradation.

In agreement with QCMD measurements, the DPI data also revealed the minimal effect of a 0.01 U mL^{-1} polygalacturonase solution on the PLL–PGA polymer mass and thickness regardless of the number of layers. Although the comparison of the enzymatic hydrolysis of 10 and 11-layer PLL–PGA films, when exposed to a polygalacturonase solution of 0.01 U mL^{-1}, demonstrated that the 11-layer PLL–PGA film showed a greater loss of mass when compared to that observed for a 10-layer PLL–PGA film in response to 0.01 U mL^{-1} polygalacturonase solution, it is clear that this effect was to a major extent caused by a change in pH rather than being due to the enzyme action. As noted earlier, the exposure of 10 and 11-layer PLL–PGA films to pH 4.0 buffer solution resulted in greater changes in mass to PLL–PGA films with PLL as the top layer. FTIR-ATR measurements on these multilayers showed that in PGA–PLL multilayers assembled at pH 7.0 the PGA protonation on decreasing pH to 4.0 is inhibited by the strong polyanion–polycation interactions (Westwood et al., 2010, in press). Once the multilayer is weakened by the depolymerisation of the PGA these interactions will be weakened and the PGA protonation will no longer be inhibited. Further electrostatic interactions may occur between the enzyme and the polycation. As the IEP of the enzymes are in the range 4.1–5.0 it is possible that some of the PLL stripped from the surface may complex with the enzyme, further contributing to the arrest of hydrolysis at low enzyme concentrations (Gasteiger et al., 2003).

It is important to mention that an enzyme–substrate complex must be formed in order to initiate enzyme action on the substrate and as Sasaki et al. (2008) reported for α-amylase hydrolysis of potato amylopectin an increase in polymer mass prior to loss due to hydrolytic action. However in our case we believe that the immediate rapid hydrolysis occurred upon addition of enzyme, and thus we did not observe an initial increase in hydrated mass with either the QCMD or the DPI measurements. The enzyme concentrations (0.0006–0.06 mg mL^{-1}) are at least a factor of ten lower than those used in deposition of the multilayers and, in cases in which hydrolysis is relatively rapid, an increase in polymer mass due to enzyme adsorption will not be observed. The findings presented here contrast conclusions with those of Akashi et al. (Serizawa et al., 2002). They demonstrated that chitosan–dextran sulphate multilayers, with dextran sulphate as a top layer, were hydrolysed faster with chitosanase when compared to the same film having chitosan as the top layer, even though chitosanase hydrolyses chitosan. It was proposed that this unique hydrolysis resulted from electrostatic condensation of chitosanase on top of the dextran sulphate layer subsequently leading to hydrolysis of chitosan in the underlayer. This, however, does not appear to take place in the case of polygalacturonase and PLL–PGA films. Investigation of the effect of the deposited top PLL layer on the rate of degradation showed that the PLL top layer slowed enzymatic hydrolysis of PLL–PGA films. Although the PLL coating did not prevent the degradation, it is considered to modulate the access of the enzyme to PGA, possibly providing a barrier to hydrolysis. It is considered that when a significant amount of PGA has been desorbed from the films, the amount of PLL that remained had collapsed blocking the penetration of the polygalacturonase into the remaining structure and thus slowing degradation.

3.2.3. AFM

The AFM topographic images of PLL–PGA films taken before the hydrolysis showed that films were sufficiently rigid to allow imaging of the molecular structure (Fig. 6A and B). Both 10 and 11-layer films appeared as heterogeneous fibrous networks containing aggregated structure with surface roughness measurements (root mean square, RMS) being 5.6 nm and 3.27 nm for 10 and 11-layer PLL–PGA film, respectively. The AFM images taken after the enzyme hydrolysis clearly showed the effect of the hydrolysis on both 10 and 11-layer films (Fig. 6C and D). The enzyme action led to a similar final outcome regardless of the number of layers. Due to the enzymatic hydrolysis of PGA, both films lost their continuous fibrous structures that were formed during the assembly of 10 and 11-layer PLL–PGA films, and the remaining structure appeared to show greater amount of aggregates. The RMS values that were taken after the hydrolysis were, however, lower and estimated to be 2.04 nm and 1.74 nm for the 10 and 11-layer PLL–PGA assembly, respectively. Lower RMS values and visible changes in the films structures imaged with AFM and the results obtained using QCMD and DPI clearly confirmed partial disassembly of PLL–PGA films in response to hydrolysis.

Although by the monitoring changes in polymer mass and optical thicknesses we have confirmed the conclusions drawn based on QCMD measurements, indicating that the top PLL layer has slowed the biodegradation of PLL–PGA films, this was not apparent from AFM data. Nevertheless, in accord with the QCMD and DPI results shown earlier, the action of 1 U mL^{-1} polygalacturonase solution on 10 and 11-layer films led to the same final outcome and the AFM images shown here appear to support that.

4. Conclusions

PLL–PGA multilayer thin films were constructed and tested at human physiological temperature of 37 °C. After the multilayers were formed the degradation of PGA by polygalacturonase was monitored with QCMD, DPI, and AFM. PLL a cationic poly-amino acid was used as the polycation in order to assemble a polyelectrolyte multilayer with the polyanionic PGA. Using PLL as a multilayer constituent resulted in an exponential growth regime due to the diffusion of PLL within the PLL–PGA multilayer film during its formation, leading to a successive increase of mass and thickness of the composite with each deposition step. Adding polygalacturonase to PLL–PGA multilayers led to the significant reduction of the hydrated and polymer mass of PLL–PGA multilayer films. It has been shown that the rate of degradation is concentration dependent and the initial degradation rates for different polygalacturonase concentrations were estimated. Whereas, the polygalacturonase activity of 0.01 U mL^{-1} was found to have a negligible effect on the decomposition of PLL–PGA films, at the highest enzyme activity of 1 U mL^{-1} an immediate reduction in the hydrated and polymer mass of PLL–PGA multilayer films was

observed. Investigation of the effect of the deposited top PLL layer on the rate of degradation showed that the PLL top layer slowed enzymatic hydrolysis of PLL–PGA films. Although the PLL coating did not prevent the degradation, it is considered to modulate the access of the enzyme to PGA, possibly providing a barrier to digestion. The significant impact of polygalacturonase concentration was a fundamental outcome of this research and proved that the presence of diluted polygalacturonase resulted in enzymatic hydrolysis of polygalacturonic acid chains and consequently almost complete decomposition of the multilayer film (loss of over 80% of the total mass) at polygalacturonase concentration higher than 0.1 U mL^{-1}. However, there is potential to modulate the rate of degradation, and thus the stability of PLL–PGA films in response to enzymatic degradation, by an appropriate coating of the film.

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