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Facilitating high-force single-polysaccharide stretching using covalent attachment of one end of the chain

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ABSTRACT

Single polysaccharide force spectroscopy has yielded particularly interesting data, the interpretation of which requires the marriage of statistical–mechanical theories of polymer physics to the complexities afforded by possible force-induced conformational transitions of the constituent sugar rings. However, the difficulty of designing handles for the specific attachment of the different ends of polysaccharide chains to substrates, such as piezoelectric scanners, cantilevers or microbeads has meant that the majority of studies to date have been carried out with the polymer physisorbed to the substrates between which it is stretched, or at best chemically attached via bonds formed at uncontrolled locations along the length of the molecule. This means that the lengths of obtained polysaccharide stretches, as well as the forces that can be placed on the molecule without generating detachment, are generally smaller than those obtainable for polymers that offer the ability to be covalently attached to substrates specifically at their ends. As a consequence it is troublesome and tedious to record a statistically significant number of force curves that extend chains to high enough forces to investigate certain conformational transitions, such as the boat-to-inverted chair, exhibited by polysaccharides such as pectin. Herein, single molecule force–extension curves have been measured for the several pectin samples using AFM. The results are compared when either (1) the polymers have been physisorbed between the cantilever and the surface of the piezo-electric scanner, under several different solvent conditions of pH and ionic strength, or (2) the polymer molecule has been chemically attached at one end to the piezo surface using a recently reported coupling procedure. In fact, using such a chemical attachment to tether the end of the polysaccharide, reduced the frequency of successful stretching events obtained in a particular location, confirming the role of surface diffusion in the physisorbed experiments. Nevertheless, when polymer stretches were successfully recorded, the force that could be applied before detachment was significantly increased, indicating that this methodology has great potential for improving the acquisition of data reporting on force-induced conformational transitions of the sugar ring that require the application of significant stresses.

1. Introduction

Modern biophysical instrumentation allows forces in the pN–nN range to be applied to molecular chains and their displacements to be controlled and measured to nm precision. However, making controllable connections between the active nano-manipulators (cantilevers, beads or piezo-ceramics) and the molecules of interest are key, not only to facilitate force–extension measurements, but also in expanding these tools into the realms of more sophisticated experiments such as those monitoring interactions and real-time kinetics. While nucleotides and proteins both intrinsically present termini that have distinct chemistries at each end of the chain and have manipulation possibilities afforded by the developed tools of molecular biology (Bustamante, Smith, Liphardt, & Smith, 2000; Kellermayer, Smith, Granzier, & Bustamante, 1997) developing such "handles" for the end residues of polysaccharides presents more of a challenge.

Certainly great progress has already been made in single polysaccharide force spectroscopy and work carried out with the atomic force microscope (AFM) has yielded particularly interesting

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Table 1
The characteristics of the pectin samples used; degree of methylesterifications (±3); galacturonic acid content (±5); and molecular weight as quoted by the manufacturer.

Pectin	Origin	DM (%)	GA (%)	M_w (kDa)
LM 12	CP Kelco (Denmark)	35	90	130
Kelco 8A	CP Kelco (Denmark)	65	87	150
Apple pectin	Fluka Biochemica (Switzerland)	78	90	30–100
P9561	Sigma–Aldrich (St. Louis, USA)	90	≥75	∼31

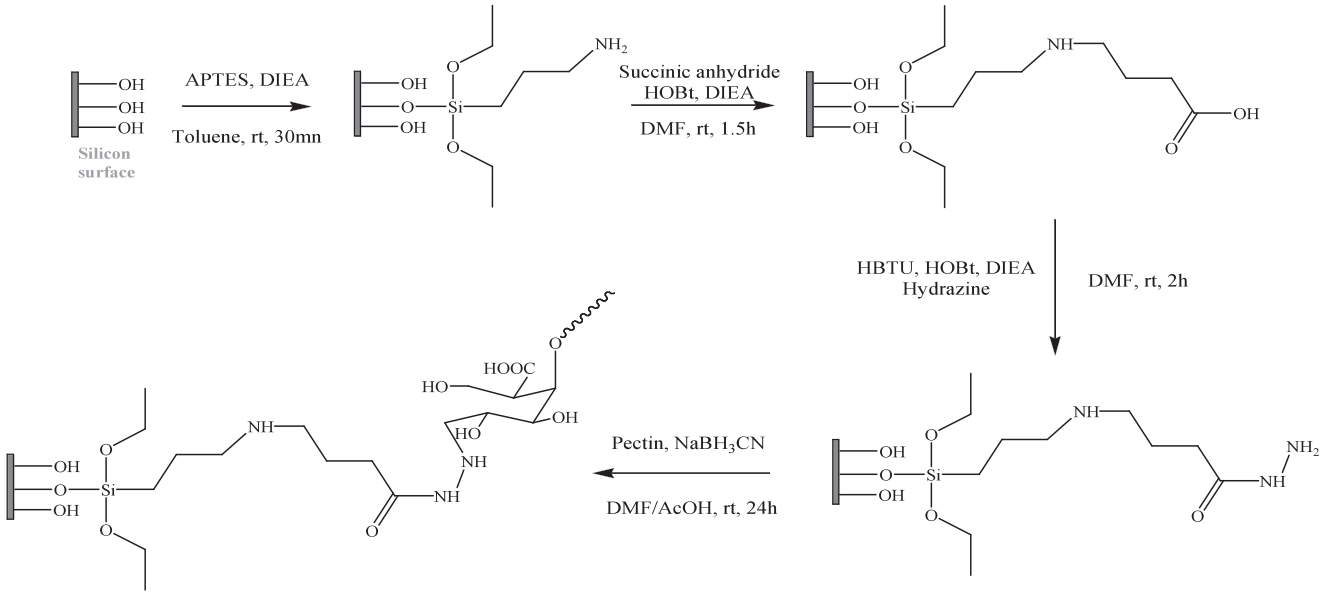

Fig. 1. A schematic of the scheme developed herein for the covalent attachment of the terminal sugar residue of pectin to hydrazide-functionalized surfaces via reductive amination (RA).

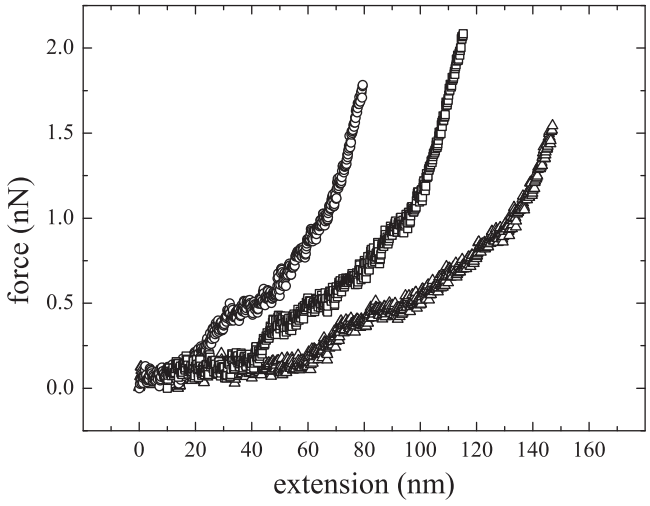

Fig. 2. Typical single polysaccharide force–extension curves measured for apple pectin. The effects of conformational transitions may be seen as "clicks", deviations from the standard monotonic wormlike chain model.

data, the interpretation of which requires the marriage of statistical–mechanical theories of polymer physics to the complexities afforded by possible force-induced conformational transitions of the constituent sugar rings (Haverkamp, Marshall, & Williams, 2007; Haverkamp, Williams, & Scott, 2005; Marszalek et al., 1999; Marszalek, Li, & Fernandez, 2001; Marszalek, Li, Oberhauser, & Fernandez, 2001; Zhang, Wang, Cui, Wang, & Zhang, 2003). Such monomer transitions during stretching, from classical chair forms of the pyranose ring to more elongated arrangements increase the polymer's contour length and thus produce characteristic deviations (or "clicks") in the slope of the force–extension curve. However, owing to the difficulty of designing handles for the specific attachment of the different ends of polysaccharide chains as described, the majority of studies have been carried out with the polymer in question physisorbed to the substrates between which it is stretched, with a minority held through physical interactions between covalently attached binding pairs (Marszalek, Oberhauser, Pang, & Fernandez, 1998), or at best with one contact chemically attached to a substrate via a covalent bond (Khner, Erdmann, & Gaub, 2006), but at an uncontrolled location along the length of the molecule.Polysaccharide stretching studies to date then have been largely focused on AFM and on picking up lengths of physisorbed polymers by chance from different surfaces. Such work typically attempts to locate the most favorable solution conditions for achieving stretches with desired properties (Maurice & Matthai, 1999). Difficulties are exacerbated by a lack of knowledge about the nature of the physisorbed contact points. Physisorption on surfaces is generally considered to be weak, owing to the intrinsic lack of strength of physical forces such as the van der Waals forces, and many studies report the peeling of molecules from surfaces. Indeed adhesion and desorption of polymers from surfaces have become of great interest in their own right (Cui, Lui, Zhang, Zhang, & Wu, 2003; Francius et al., 2009; Geisler et al., 2008; Geisler, Horinek, & Hugel, 2009; Hugel et al., 2001; Kierfeld, 2006; Seitz, Friedsam, Jostl, & Gaub, 2003; Sonnenberg, Billon, & Gaub, 2008; Staple, Geisler, Hugel, Kreplak, & Kreuzer, 2011). If stretching is rapid enough interactions can persist until higher forces (Ray, Brown, & Akhremitchev, 2006; Ray, Brown, & Akhremitchev, 2007; Sletmoen, Skjak-Braek, & Stokke, 2004; Sonnenberg, Parvole, Kuhner, Billon, & Gaub, 2007); and if pinning is induced by a surface feature of the substrate or by another overlapping chain then occasionally a contact is made

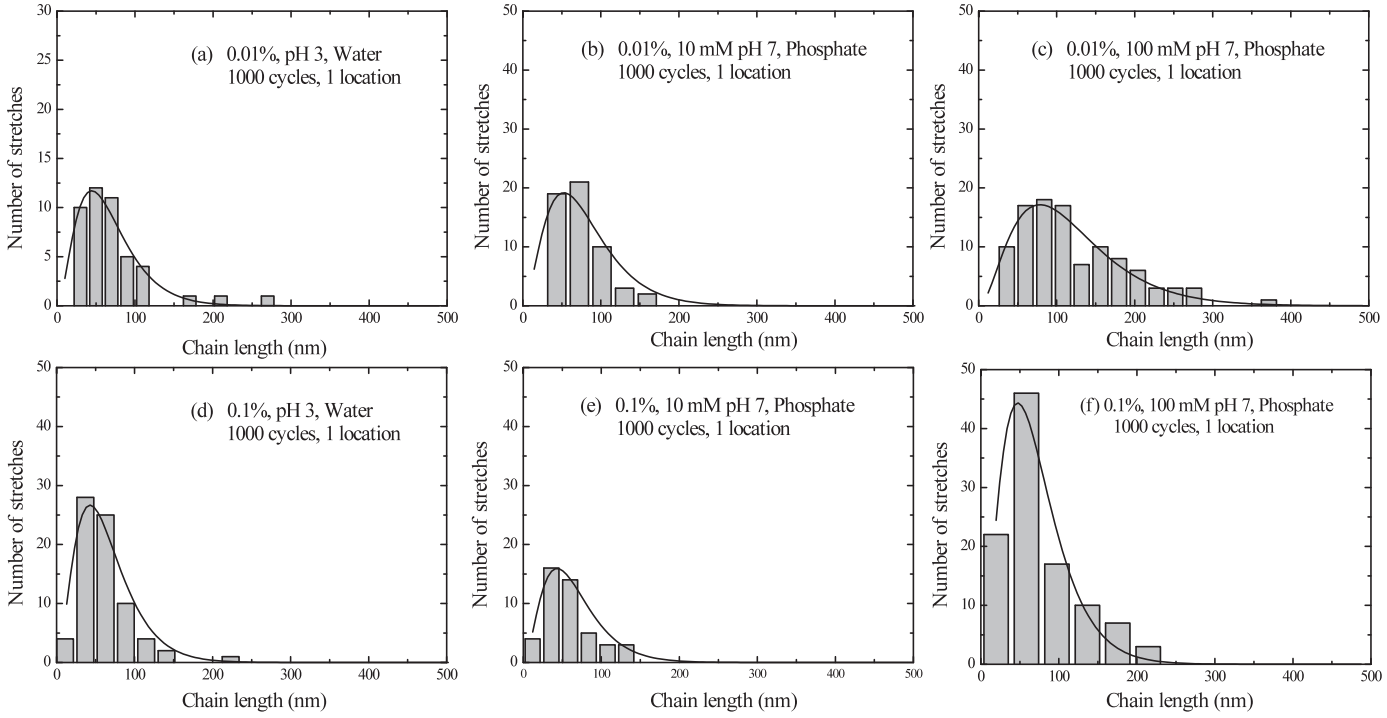

Fig. 3. Distribution of single-molecule detachment lengths found for LM 12 pectin in different experimental conditions: (a) 0.01% polymer, pH 3 (b) 0.01% polymer, pH 7 10 mM (c) 0.01% polymer, pH 7 100 mM (d) 0.1% polymer, pH 3 (e) 0.1% polymer, pH 7 10 mM (f) 0.1% polymer, pH 7 100 mM. The solid lines are fits to the data described in the text.

that is strong enough to suffice for a significant stretch to ensue. In general however, when such a methodology is applied, a large number of scans can be needed in order to achieve an effective stretch, involving tedious trials of several solvent conditions, and making it particularly difficult to perform, for example, multiple scanning and stretch reversal studies.

While for AFM studies specifically covalently attaching both ends of a polysaccharide molecule to a substrate and cantilever tip respectively remains the ultimate goal, a methodology has recently been reported that makes some progress in this direction by convincingly demonstrating, using spectroscopy supported by quantum chemical calculations, the specific covalent bonding of one end of a pectin chain to microbeads (Fellah, Anjukandi, Hemar, Otter, & Williams, 2011). Pectin can be considered for the purposes herein as a polymer of alpha 1–4 linked galacturonic acid sugar rings, each of which is capable of carrying a methyl group as an ester at the C-6 position (Ralet & Thibault, 2002). This polysaccharide was selected for study here as it presents two force-induced conformational transitions upon straining that make it a particularly interesting case (Williams, Marshall, Anjukandi, & Haverkamp, 2007). Furthermore, one of these transitions, hypothesized to be a boat-to-inverted chair transition, occurs at reasonably large forces (>700 pN), making this feature difficult to observe in experiments in which the polymer is physisorbed between contacts. Herein, this recently reported coupling methodology was supplemented and applied in order to covalently link one of the terminal sugar residues of the pectin chains onto substrates that were subsequently mounted onto a piezo-electric scanner. Attempts were then made to physisorb the trailing end of the chain to an AFM cantilever using advance and retract cycles of the piezo in the usual fashion and the results obtained from a statistical sample of stretch attempts were compared when using (i) purely physisorption of both ends under different solvent conditions or (ii) where the one end was specifically chemically bound to the piezo-surface as described.

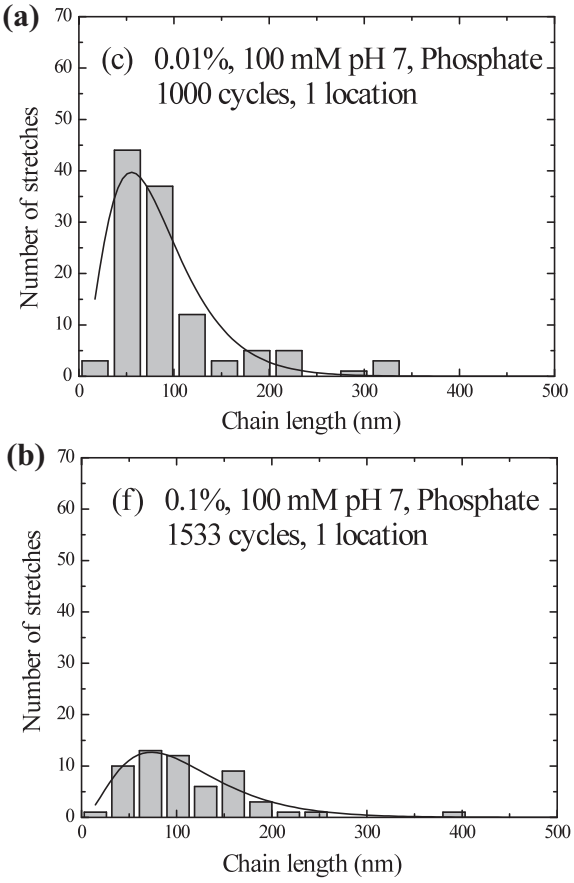

Fig. 4. Distribution of single-molecule detachment lengths for Kelco8A pectin in different experimental conditions: (a) 0.01% polymer, pH 7, 100 mM (conditions, c), (b) 0.1% polymer, pH 7, 100 mM (conditions, f). The solid lines are fits to the data described in the text.

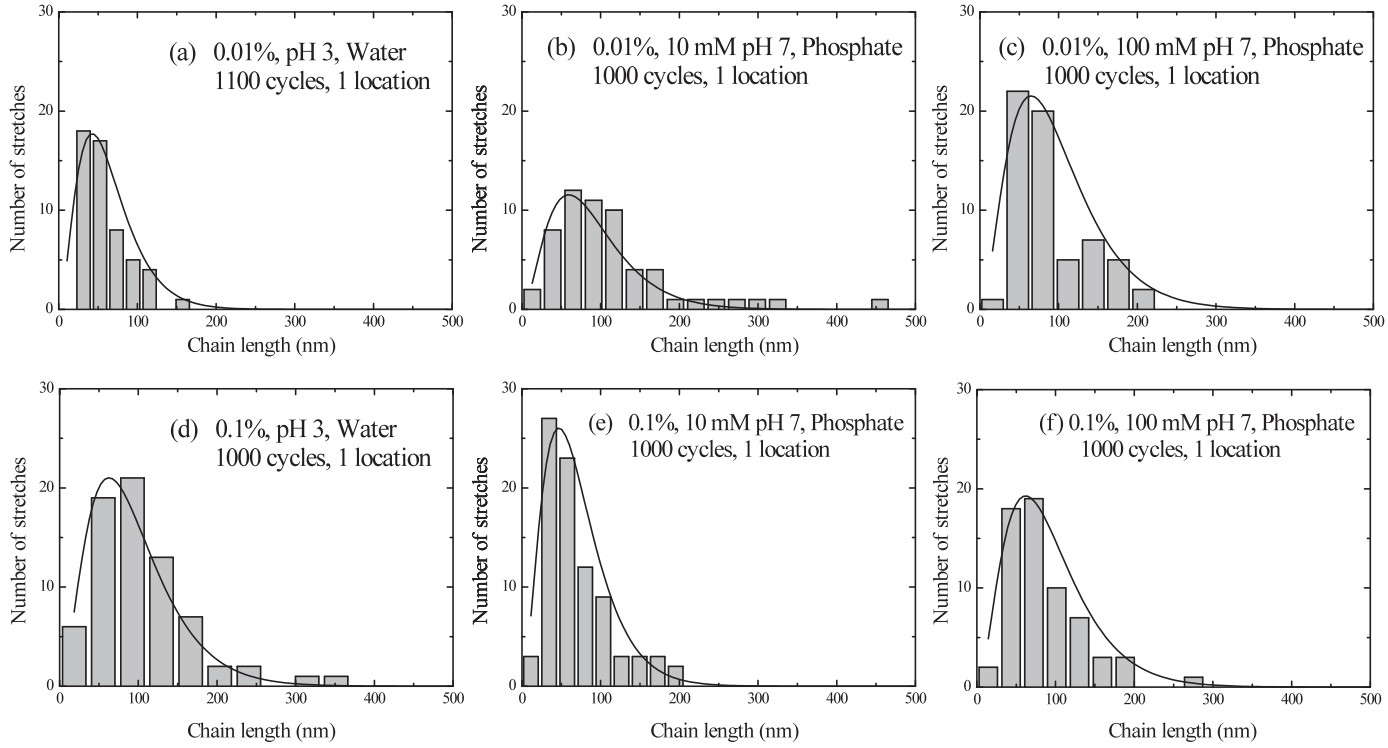

Fig. 5. Distribution of single-molecule detachment lengths for apple pectin in different experimental conditions: (a) 0.01% polymer, pH 3 (b) 0.01% polymer, pH 7 10 mM (c) 0.01% polymer, pH 7 100 mM (d) 0.1% polymer, pH 3 (e) 0.1% polymer, pH 7 10 mM (f) 0.1% polymer, pH 7 100 mM. The solid lines are fits to the data described in the text.

2. Experimental

2.1. Materials

Pectin samples with different fine structures were used, the origin, the degree of methylesterification (DM), the anhydrogalacturonic acid content (GA) and the molecular weight (M_w) of which are given in Table 1. All other chemicals were purchased from Acros Organics except Boc-Cys(trt)-OH from Bachem, and hydrazine monohydrate from Alfa Aesar. Water used throughout this study was purified with a MilliQ water purification system (Millipore) giving a water resistivity 18.2 MΩ cm. Circular glass coverslips were supplied by Warner Instruments LLC and used as substrates onto which the polymer chains were attached. AFM experiments were performed using gold tipped silicon nitride cantilevers (Ultrasharp CSG11/Au, NT-MDT Co). (Additional preliminary scouting experiments found similar behaviour to that reported here for physisorbed experiments when using mica and gold-coated surfaces.)

2.2. Polymer coupling

2.2.1. Pre-treatment

The preparation and the cleanliness of substrates were found to be of critical importance in these experiments. Prior to pectin physisorption or chemical treatment the glass cover slides were sonicated in dried toluene for 10 min, cleaned in freshly prepared Piranha solution (a mixture of 30% hydrogen peroxide and 70% sulfuric acid) for 1 h, rinsed in de-ionized water and toluene exhaustively, dried under vacuum and finally irradiated for 1 h in a UV/O_3 surface decontamination chamber (PSD/UV Novascan Technology). The cleaned surfaces were stored in dried toluene for further treatment as detailed below.

2.2.2. Physisorption

Samples of physisorbed polymers were prepared by applying 20 μL of 0.1 or 0.01% w/w pectin solution in de-ionized H_2O to cleaned discs, which were then dried at 11.3% relatively humidity overnight. These were then extensively rinsed with de-ionized H_2O leaving only tightly bound molecules. These glass substrate discs were mounted on the piezo-electric ceramic of the AFM and a "liquid cell", consisting of a perspex chamber sealed with an O-ring, and containing the pre-mounted cantilever, was placed on top of the substrate. Several channels in the wall of the chamber allowed different solvents to be introduced above the sample in situ, in which the stretching experiments were conducted. Measurements were made in de-ionized water at pH 3, or in 10 mM or 100 mM phosphate buffer at pH 7.0 as described in more detail below.

2.2.3. Chemisorption

In order to prepare the glass substrate for the ensuing attachment chemistry 3-aminopropyltriethoxysilane (APTES) films were prepared on the surface by incubating clean slides in a fresh APTES solution (1% APTES, 99% dried toluene) under sonication and in the presence of 1,1-diisopropylethylamine (DIEA), for 30 min at room temperature. Subsequently, the surfaces were ultrasonically washed with anhydrous toluene twice for 10 min to remove loosely physisorbed APTES. The resultant silanized amino-terminated surfaces were subsequently rinsed with a 1 mM anhydrous acetic acid solution and used in the following chemical coupling scheme.

The immobilization of pectins onto surfaces was carried out via a reductive amination (RA) reaction previously described for use with oligogalacturonides, (Guillaumie, Thomas, & Jensen, 2002) and more recently for polymers (Fellah et al., 2011) using microbeads as substrates. Herein the reaction is carried out in order to attach pectin molecules to flat substrates, specifically at the reducing end of the chains. The freshly amino-terminated substrates prepared

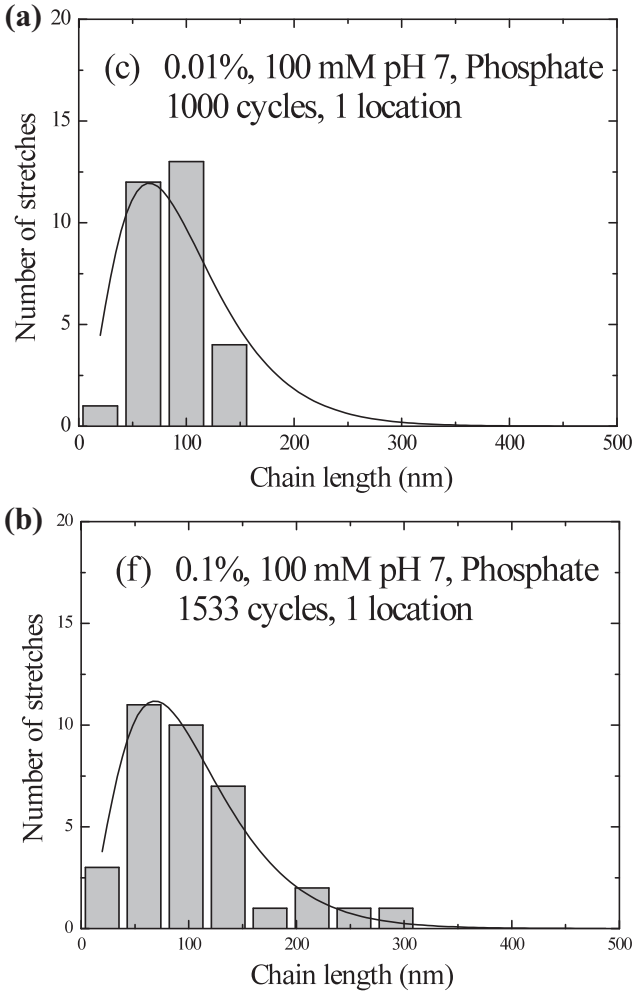

Fig. 6. Distribution of single-molecule detachment lengths for P9561 pectin in different experimental conditions: (a) 0.01% polymer, pH 7, 100 mM (conditions, c), (b) 0.1% polymer, pH 7, 100 mM (conditions, f). The solid lines are fits to the data described in the text.

as above were washed with dimethylformamide (DMF). Then succinic anhydride (30 mg) and hydroxybenzotriazole (HOBt) (30 mg) were dissolved in DMF (5 mL) with DIEA (70 μL) and this solution was added to the substrates and subsequently ultrasonicated at room temperature for 1.5 h. Thus carboxylic acid-terminated substrates are formed and were then washed thoroughly with DMF. Next, O-benzotriazole-NNN′N′tetramethyl-uronium hexafluorophosphate (HBTU) (70 mg) and HOBt (120) were dissolved in DMF (5 mL) with DIEA (180 μL) and hydrazine monohydrate (60 μL). This mixture was then added to the carboxylic acid-terminated surfaces and ultrasonicated. After 2 h coupling at room temperature, solvents and unreacted compounds were removed and the surfaces washed thoroughly with DMF, leaving the surfaces hydrazide-terminated. Pectin was dissolved in DMF/AcOH (v/v = 99:1) at 0.5% w/w. Subsequently 5 mL of this solution was added to the hydrazide-terminated surfaces and, following addition of $NaBH_3CN$ (50 mg), the mixture was ultrasonicated at room temperature. After 24 h, solvent and excess reagents were removed and the substrates were washed with DMF and dried overnight under reduced pressure. The entire procedure is shown schematically in Fig. 1. After preparation the sample was mounted in the AFM and the liquid cell described above was filled with the appropriate solvent just prior to carrying out the force-curve measurements.

2.3. AFM

Force–distance curves were recorded by pulling the molecules at 0.5–4 $\mu m\,s^{-1}$ using the scanning probe microscope head from a Veeco Nanoscope E, with a home-built laptop-based controller driving the stepper-motor and the piezoelectric scanner, based on a feedback-signal generated from a Quadrant Photodiode (QPD) (Belmiloud, Fellah, Haverkamp, & Williams, 2011). Briefly, *I*/*O* voltages were generated and recorded simultaneously, every microsecond. A voltage booster was constructed in order to increase the available voltage of the output signal, without supplying appreciable power, which preserved *I*/*O* synchronization and gave a control system with a spatial resolution estimated at 1 nm.

Prior to each experiment cantilevers were cleaned in a mixture of ammonia and hydrogen peroxide (v/v = 2:1) for 1 h, rinsed thoroughly with de-ionized water and absolute ethanol, dried under a stream of nitrogen gas and finally exposed to ultraviolet light in a UV/O_3 decontamination chamber for 30 min. They were then calibrated using the well-documented thermal method (Sader, Pacifico, Green, & Mulvaney, 2005). This measurement was made in air so the first resonance of the cantilever was not damped and was easily isolated from the other modes. Gold-coated cantilevers were preferred and maximized the QPD sensitivity. Typical cantilever force constants were 50 $pN\,nm^{-1}$.

In order to facilitate the acquisition of a large set of experimental data a custom-designed software protocol was written in LabView that allowed on the-fly-filtering of the dataset, as reported elsewhere (Belmiloud et al., 2011). Briefly, the first step of analysis was to obtain the cantilever deflection in nm from the QPD signal, and then from this deflection, the force in nN was obtained using the spring constant of the cantilever as obtained from the thermal calibration method. To calibrate the deflection it was assumed that, in an absence of deformation of the sample, and neglecting a light elastic hysteresis of the piezoceramic, that when the piezo-mounted substrate contacts the cantilever and subsequently bends it before retraction, (the compliance region), that the deflection of the cantilever is equivalent to the distance traveled by the piezo. With the slope of the compliance region and the spring constant of the cantilever in hand, force–extension curves can be measured. The developed protocol detects the compliance region automatically and also monitors localized jumps in the force curve, assessing local standard deviations in a sliding window and comparing them statistically with the baseline and user-defined confidence intervals. Thus the user can be notified if likely single-molecule stretching events occur and data that correspond to possible events can be saved for post-run processing. Additionally the developed software reports the detachment force and the approximate value of the stretch length.

3. Results and discussion

In the present work coupling chemistry was developed to permit the direct covalent attachment of pectin to glass surfaces and was accomplished by first functionalizing the surface with amine groups. These were ultimately modified to hydrazide groups that were subsequently used to attach specifically the reducing end of pectin molecules via reductive amination. Starting with the introduction of an amine functionality onto the surface is the most widely used approach to functionalizing solid supports with biomolecules as amines have high nucleophilicity and a wide variety of amine-based coupling chemistries exist. In order to attach the prerequisite amine groups to the surface the use of APTES, widely used as a coupling agent in order to enhance the adhesion between polymeric matrices and inorganic solids (Crampton, Bonass, Kirkham, & Thomson, 2005), was investigated. A general

Table 2
Single molecule stretch experiments: polymer; concentration; solvent conditions; attachment (physisorbed (P), covalently end-tethered (C)); event frequency; probability of residue adhesion, and attachment energy.

Polymer	Polymer cone (%)	Conditions of experiment	Attachment method	Stretch frequency (%)	Attachment probability	Binding energy (k_BT)
LM12	0.01	pH 3 (a)	P	5	0.093 ± 0.004	2.27 ± 0.05
LM12	0.1	pH 3 (d)	P	8	0.095 ± 0.004	2.25 ± 0.05
LM12	0.01	pH 7, 10 mM (b)	P	5	0.079 ± 0.005	2.45 ± 0.06
LM12	0.1	pH 7, 10 mM (e)	P	5	0.095 ± 0.002	2.25 ± 0.03
LM12	0.01	pH 7, 100 mM (c)	P	10	0.053 ± 0.004	2.88 ± 0.05
LM12	0.1	pH 7, 100 mM (f)	P	10	0.087 ± 0.003	2.34 ± 0.04
LM12	0.1	pH 7, 100 mM (g)	C	10	0.055 ± 0.003	2.84 ± 0.04
Kelco8A	0.01	pH 7, 100 mM (c)	P	11	0.075 ± 0.005	2.50 ± 0.06
Kelco8A	0.1	pH 7, 100 mM (f)	P	4	0.057 ± 0.002	2.80 ± 0.03
Kelco8A	0.1	pH 7, 100 mM (g)	C	10	0.055 ± 0.004	2.84 ± 0.05
Apple	0.01	pH 3 (a)	P	5	0.097 ± 0.006	2.22 ± 0.08
Apple	0.1	pH 3 (d)	P	7	0.067 ± 0.004	2.62 ± 0.05
Apple	0.01	pH 7, 10 mM (b)	P	6	0.069 ± 0.005	2.59 ± 0.06
Apple	0.1	pH 7, 10 mM (e)	P	8	0.089 ± 0.005	2.32 ± 0.06
Apple	0.01	pH 7, 100 mM (c)	P	6	0.065 ± 0.004	2.66 ± 0.05
Apple	0.1	pH 7, 100 mM (f)	P	6	0.067 ± 0.003	2.62 ± 0.04
Apple	0.1	pH 7, 100 mM (g)	C	10	0.053 ± 0.006	2.88 ± 0.08
9561	0.01	pH 7, 100 mM (c)	P	3	0.063 ± 0.006	2.69 ± 0.08
9561	0.1	pH 7, 100 mM (f)	P	4	0.061 ± 0.002	2.73 ± 0.03
9561	0.1	pH 7, 100 mM (g)	C	15	0.055 ± 0.006	2.84 ± 0.08

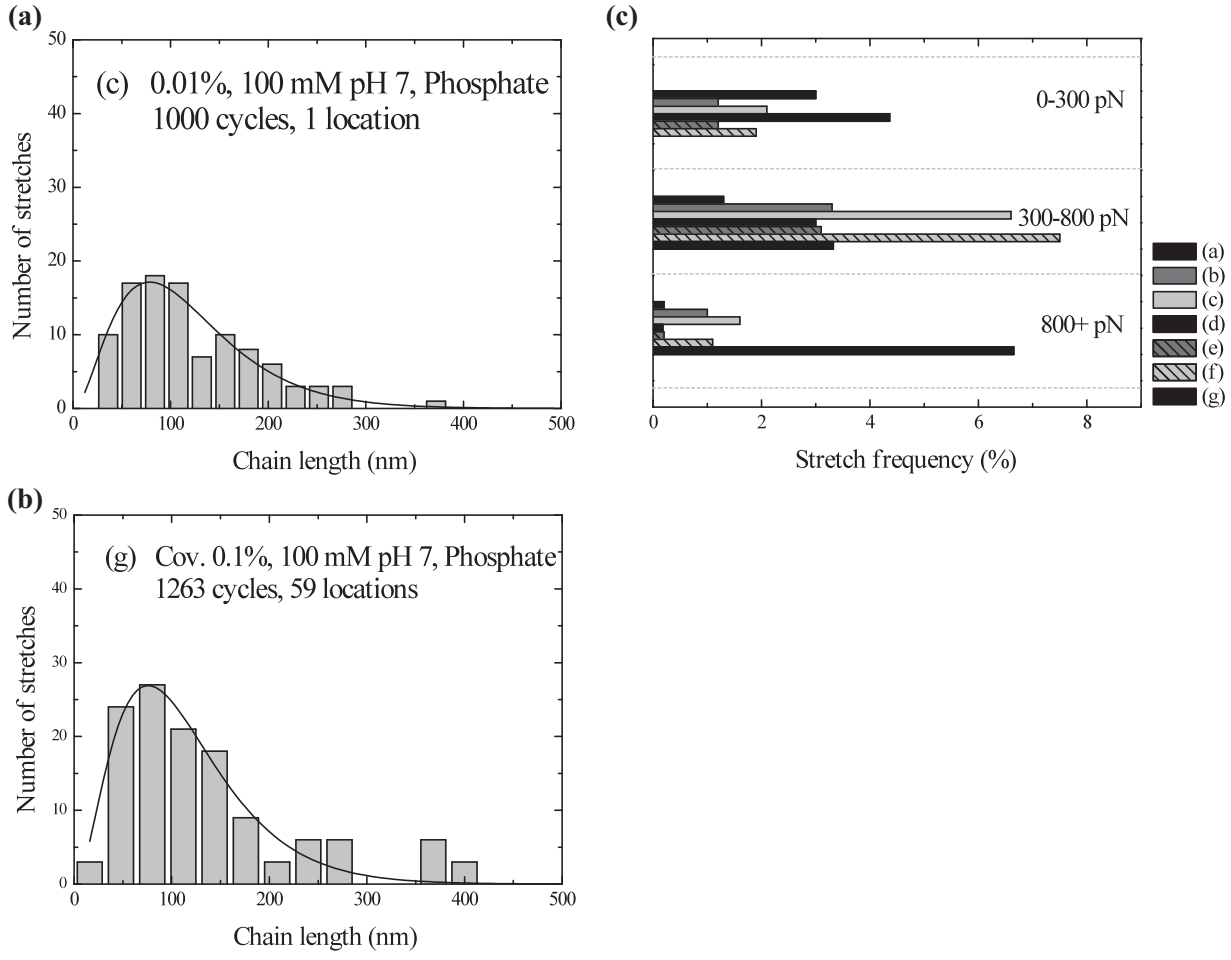

Fig. 7. (a) Distribution of single-molecule detachment lengths obtained from experiments carried out on LM 12 pectin when physisorbed (conditions, c); (b) when covalently attached (conditions, g), under the same optimum solvent conditions investigated (100 mM phosphate buffer (pH 7)); and (c) the distribution of forces achieved at detachment for the same pectin under both physisorbed and covalent attachment conditions. The labeling reflecting the physisorbed conditions is constant throughout (i.e. see Fig. 3).

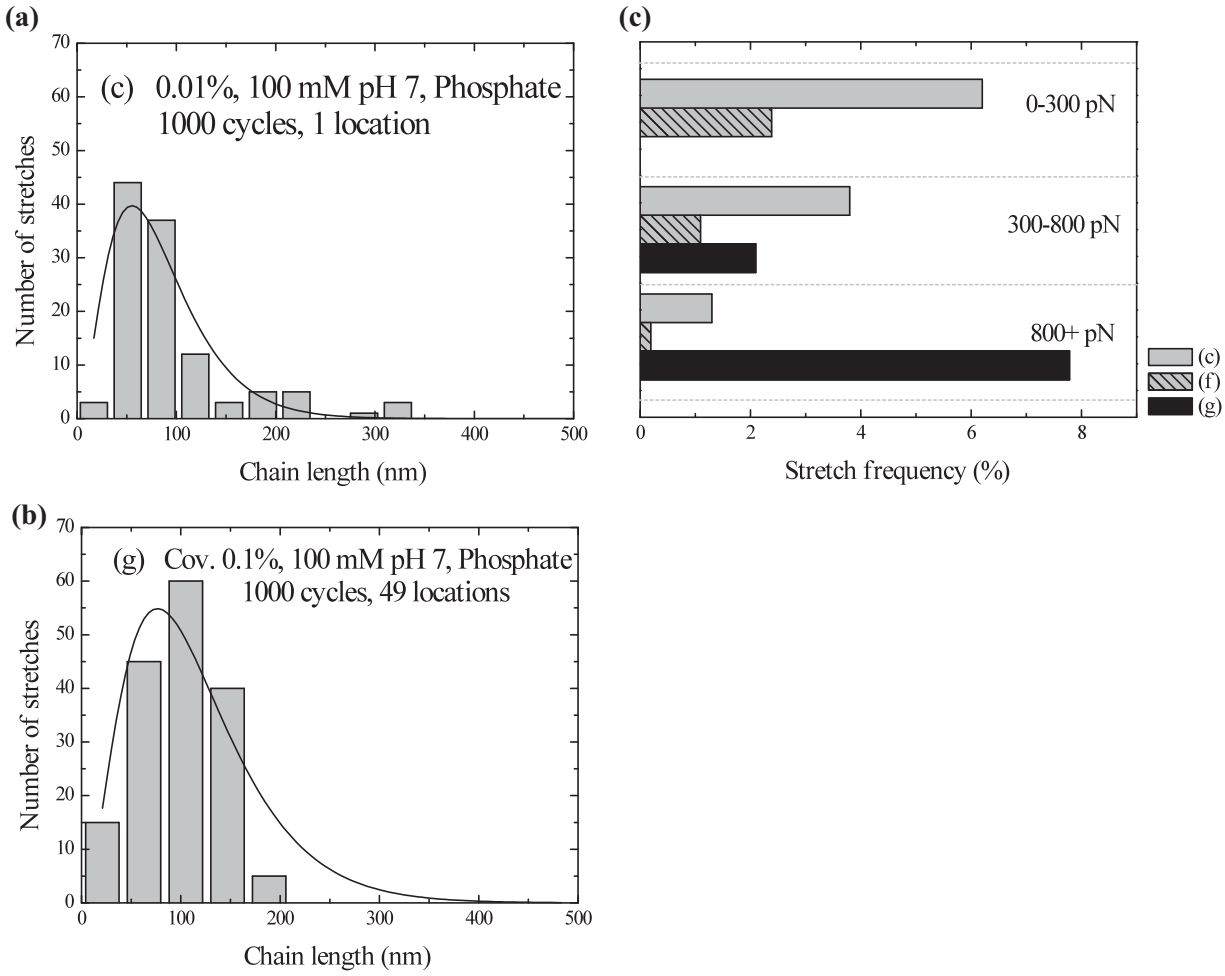

Fig. 8. (a) Distribution of single-molecule detachment lengths obtained from experiments carried out on Kelco8A pectin when physisorbed (conditions, c); (b) covalently attached (conditions, g), under the same optimum solvent conditions investigated (100 mM phosphate buffer (pH 7)); and (c) the distribution of forces achieved at detachment for the same pectin under both physisorbed and covalent attachment conditions.

consensus regarding APTES film formation on a silica substrate holds that silanization begins with the hydrolysis of ethoxy groups in APTES, a process catalyzed by water. This leads to the formation of silanols, which then condense with surface silanols forming a monolayer of APTES via a lateral siloxane network in which amino groups are oriented away from the underlying surface (Howarter & Youngblood, 2006; Simon, Cohen-Bouhacina, Porte, Aime, & Baquey, 2002). It is well known that the properties of the resultant APTES films are affected by preparation conditions such as the cleaning procedure, the choice of reaction solutions (APTES concentration, solvent) and the deposition time. It was important to ensure that APTES deposited as a smooth uniform thin film and did not experience aggregation at the surface or the deposition of multi-layers. Different combinations of the following parameters were therefore trialed during the work reported herein: cleaning procedures (HCl/H_2SO_4, MeOH/HCl, UV/O_3), solvents (toluene, chloroform, ethanol), APTES concentrations (1%, 5%), reaction times (30 min, 1 h, 24 h), and temperatures (20 °C, 60 °C).

In brief, it was found that the wet cleaning methods employed effectively cleaned the surfaces and produced a highly hydrophilic interface. DIEA promoted the hydrolysis of hydroxyl groups to allow covalent siloxane bonding. The APTES deposition reaction is potentially not limited to exclusively monolayer formation but can continue through polymerization at the surface (because of the favorable head-and-tail group interactions, APTES can form zwitterions in solution and at the film surface, resulting in multilayer formation as the reaction is extended). However, using ultrasonic waves near the surface during the reaction, polymerization of the free ethoxy-groups of APTES was found to be prevented and any physisorbed material, which may hinder the chemical reaction itself, was removed. The AcOH activates the silane to form a network on the surface, and results in any silane material not strongly bound to the surface being rinsed off. In summary, a surface amination procedure was developed that ultimately resulted in good single molecule force curves (H_2SO_4/H_2O_2, toluene, 1%, 30 min and 20 °C). The other variations led to various extents, to peeling the attached layer off the surfaces, that we attributed to the formation of heterogeneous multilayers.

With a successfully optimized functionalization of glass substrates in hand experiments focused on finding the physisorption conditions that yielded the best single molecule stretches (in terms of frequency, length and detachment force), so that subsequently results obtained thus could be compared with those obtained from substrates modified as described with the terminal sugar residue at one end of the chain chemically attached to the surface.

Fig. 2 shows typical single molecule force–extension curves recorded from pectin during the course of this work. The results agree well with previous work and in particular clearly show “clicks”, the characteristic deviations in the force extension curve arising from force-induced conformational transitions in the sugar rings (Marszalek et al., 1999; Williams et al., 2007).

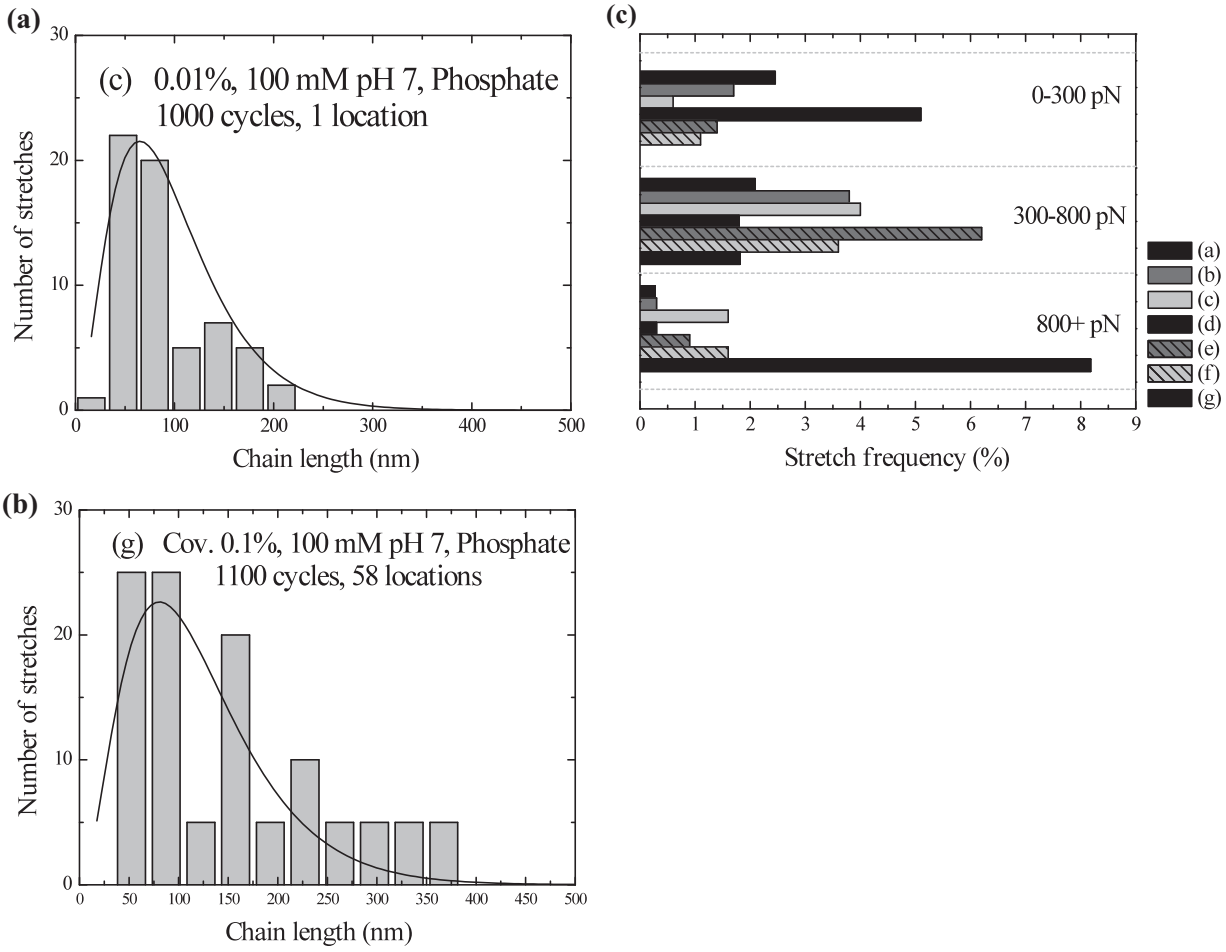

Fig. 9. (a) Distribution of single-molecule detachment lengths obtained from experiments carried out on apple pectin when physisorbed (conditions, c); (b) covalently attached (conditions, g) under the same optimum solvent conditions investigated (100 mM phosphate buffer (pH 7)); and (c) the distribution of forces achieved at detachment for the same pectin under both physisorbed and covalent attachment conditions.

Figs. 3–6 show the distribution of detachment lengths obtained from experiments carried out on pectins of ~35%, 55%, 75% and 90% DM respectively when physisorbed between glass and a gold-coated AFM tip under different solvent (de-ionized water (pH 3); 10 and 100 mM phosphate buffer (pH 7)) and preparation conditions (dried solution 0.01 or 0.1%).

Perhaps the most striking observation is the reasonably small effect that these variations in solvent conditions had on the results obtained in the range investigated. The forms of the histograms obtained are similar to those obtained from other studies on physisorbed polymers (Grandbois, Beyer, Reif, Clausen-Schaumann, & Gaub, 1999; Kocun, Grandbois, & Cuccia, 2011; Li et al., 1999; Mueller, Butt, & Bamberg, 1999; Nakajima, Watabe, & Nishi, 2006; Oritz & Hadziioannou, 1999; Valle et al., 2008; Zou, Schonherr, & Vansco, 2005). Furthermore, similar relative insensitivities to salt and pH have also been reported (Pirzer, Geisler, Scheibel, & Hugel, 2009; Roiter & Minko, 2004, 2007). While some of this previous work was focused on measuring surface desorption by continuously peeling molecules, it can be argued that the length distribution obtained in such a manner should have a similar functional form to that obtained in experiments of the type described here, with the terminal positions of the adsorbed or "picked-up" sections being determined randomly (Sonnenberg et al., 2008).

The resulting histograms have been fitted to a simple model of the form $A \times DP^2 \exp(-B \times (DP))$; with DP the degree of polymerization and A and B constants. This successfully captures the hypotheses that (a) attachment of the chain to the substrate or the cantilever tip in a manner such that a successful stretch follows is a random event and can occur at any residue with an equal probability; hence yielding an exponential distribution of stretch lengths; and (b) that the lengths should also be weighted by the possible proximity of a polymer attachment point to the AFM tip onto which it may physisorb; longer sections of chain have a larger chance to be picked up in each extend-retract cycle as they sample an area of the surface proportional to the square of their length.

It can be seen that given the limited dataset these fits provide a reasonable description of the experimental data. A similar model has previously been described (Sonnenberg et al., 2008), although it is noteworthy that the modeling of such experimental histograms has generally not been undertaken in much of the previously reported work. The modeling performed herein allows the estimation of the probability for the attachment of a single residue to the substrate from the value of the parameter *B*, and hence an estimate of the binding energy can be made. As expected from the appearance of the raw data remarkably little variation was found between the results from experiments employing different sets of conditions, with the values spanning 2.2–2.9 kT (Table 2). This corresponds to a range of 56–63 meV, which indeed falls well within the expected range for physisorption phenomena, typically between 10 and 100 meV. While it should also be remembered that, as shown in Table 2, a large number of stretch attempts did not yield a single molecule stretch, the relevant fit parameters, and thus estimates of the probabilities and binding energies that result from the analysis of the data obtained when stretches are successfully

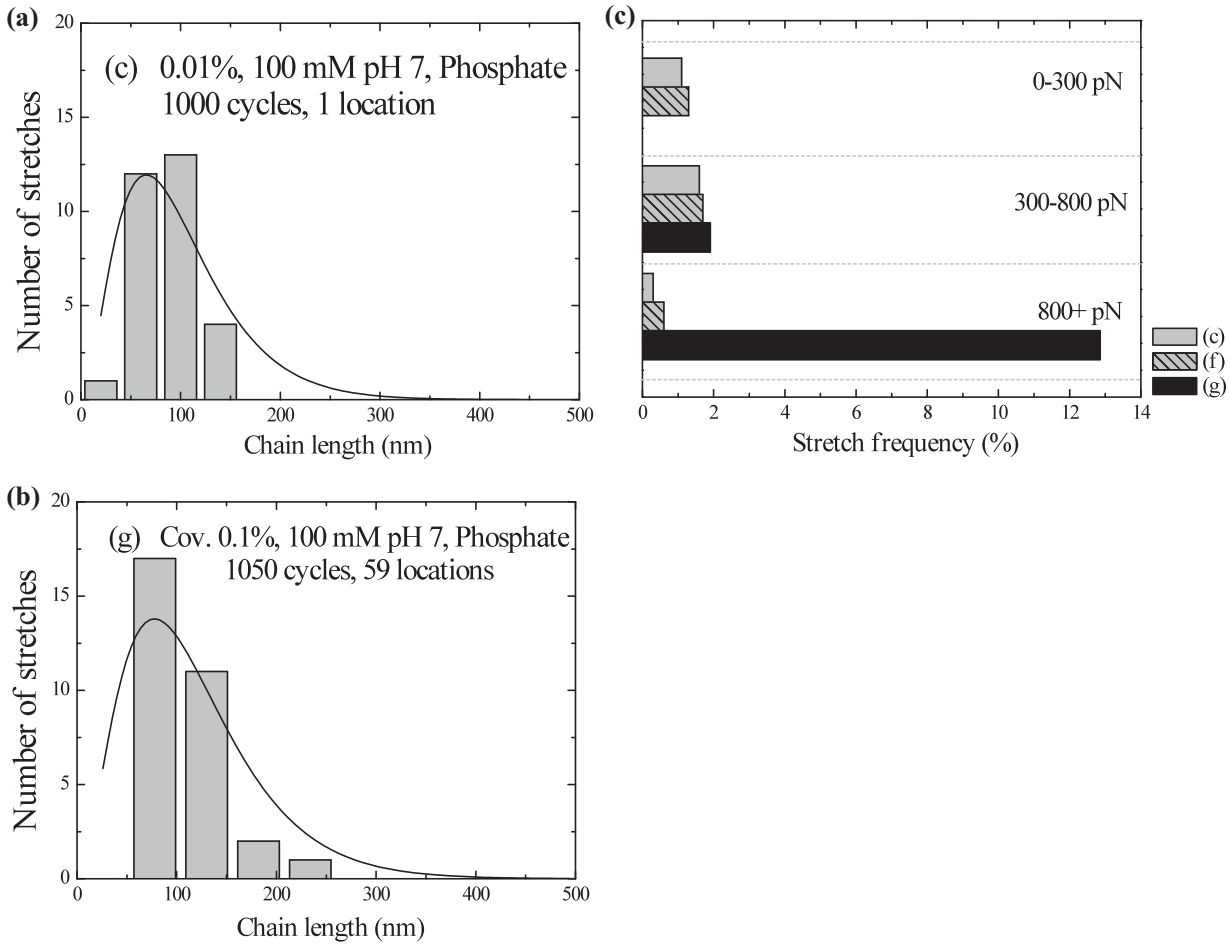

Fig. 10. (a) Distribution of single-molecule detachment lengths obtained from experiments carried out on P9561 pectin when physisorbed (conditions, c); (b) covalently attached under the same optimum solvent conditions investigated (100 mM phosphate buffer (pH 7)); and (c) distribution of forces achieved at detachment for the same pectin under both physisorbed and covalent attachment conditions.

measured, are given in Table 2. As expected, the samples yielding longer average stretch-lengths have a slightly lower probability of each residue binding, corresponding to larger energy difference between the bound and unbound states.

While the effects of the solvent and preparation conditions appear minor the trends observed are nonetheless consistent with expectations. Firstly, the effect of the concentration of polymer used to create the dried layer appears minimal. However, both the polymer concentrations used are in excess of that required to provide a monolayer, (surface coverages would be equivalent to a chain every few nm), and as such previously reported work suggesting that washing as described in the experimental section removes all but a strongly bound monolayer (Marszalek et al., 1999, 2001) would seem pertinent. This suggests that by the time the substrates are introduced into the liquid cell for experiments the coverage is likely to be the similar in both cases. Secondly, for the most charged (lowest DM) polymers the most favorable conditions for yielding stretches appear to be pH 7, 100 mM phosphate where, while both surface and polymer are negatively charged, the relatively high ionic strength facilitates attachment. Lastly, DM appears to have only a minimal effect under the conditions investigated, consistent with the screening of charge effects, but also suggesting that the presence of methylesterified residues does not play a dominant role in binding.

Figs. 7–10 show (a) the distribution of detachment lengths obtained from experiments carried out on pectins of ~35%, 55%, 75% and 90% DM respectively when physisorbed between glass and a gold-coated AFM tip under the optimum solvent conditions investigated, (100 mM phosphate buffer (pH 7)); (b) the distribution of detachment lengths obtained from experiments carried out on the same pectins in the same solvent conditions as in (a), but where the reducing end of the pectin molecule is covalently attached to the glass substrate; and (c) the distribution of forces achieved at detachment for the same pectins under both physisorbed conditions and when possessing the described chemical coupling. With the goal in mind of facilitating further study on the force-induced conformational transitions of polysaccharides the measured maximum forces applied before detachment are simply considered in three bins corresponding to forces less than 300 pN; between 300 and 800 pN; or greater than 800 pN, thus corresponding to stretches that would allow the investigation of 0, 1 or 2 of the manifest conformational transitions in pectin respectively.

The data convincingly reveal that the forces obtained before detachment are substantially larger for the chemically end-tethered chains. There is also an indication, particularly in the data shown in Figs. 7, 9 and 10, that there is a slight increase in the average lengths of the sections picked up when one end is chemically attached, as might be expected. It should however also be noted that the fits to the data from the chemically end-tethered experiments are, in general, of poorer quality compared to the physisorbed experiments owing largely to the increasingly good statistics required to reliably sample the tail of the predicted exponential distribution as the lengths increase. It is also important to state that while the data from the physisorbed experiments

was acquired from only one location per sample, the chemically attached polymers required some fifty different locations to be probed to achieve the same number of successful stretch events. It appears that tethering the end of the chain to the surface prevents surface diffusion that might well be expected in the physisorbed case (Gunning, Kirby, & Mackie, 2004) and therefore limits the number of chains that can be picked up during repeated extend-retract cycles of the AFM tip in an initially barren region of the substrate.

4. Conclusion

In summary, if maximising the frequency of successful polysaccharide stretch events in a particular spatial location of the substrate is paramount then chemically attaching both ends of the polysaccharide is still a necessary goal. However, the approach taken here by chemically tethering one-end of the chain does appear to increase the average stretched length, and furthermore dramatically improves the fraction of successful stretches that yield high forces, thus offering a method to facilitate the study of conformational transitions requiring large strains.

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