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Towards polysaccharide handles for single molecule experiments: Spectroscopic evidence for the selective covalent coupling of terminal sugar residues to desired substrates

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ABSTRACT

Single molecule force spectroscopy (SMFS) has recently given access to an unprecedented level of information regarding the stress-response of a host of biopolymers at the single chain level. 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; particularly in contrast to nucleotides and proteins, has meant that progress in more sophisticated single carbohydrate measurements has been relatively slow. Herein, methods for generating specific covalent attachments of the terminal sugar residue at one end of a pectin chain to polystyrene microspheres are demonstrated. Using FT-IR spectroscopy in combination with density functional theory (DFT) calculations the attachment was unequivocally shown to be mediated by the introduction of a C–N bond between the reducing end of the polysaccharide chain and a pre-aminated bead.

1. Introduction

In the simplest type of single molecule force spectroscopy experiment two spatially separated points on a molecule are held by two separate contacts, for example a micro-cantilever and a substrate (AFM experiments), or a pair of micron-sized beads (Optical Tweezers (OT) experiments) (Buchachenko, 2006; Butt, Cappella, & Kappl, 2005; Fisher, Marszalek, Oberhauser, CarrionVazquez, & Fernandez, 1999; Jansho, Neitzert, Oberdrfer, & Fuchs, 2000; Sarid, 1991). One of the points is then moved away from the other one in a controlled manner, typically using a piezoelectric scanner to translate the substrate or a steered laser beam to move a bead, and the force involved is directly measured via the induced displacement of a tethered cantilever or optically trapped bead. Such technologies allow pN forces to be applied to the contact points and their displacements to be controlled and monitored in the nanometer range. Making controllable connections between these nano-scale force-transducers and the molecules of interest is key, not only to facilitating simple 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 furthermore 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 been made in single polysaccharide force spectroscopy and work has yielded particularly interesting data, including the observation of force-induced conformational transitions of the constituent sugar rings (Haverkamp, Williams, & Scott, 2005; Haverkamp, Marshall, & Williams, 2007; Marszalek et al., 1999; Marszalek, Li, & Fernandez, 2001; Marszalek, Li, Oberhauser, & Fernandez, 2002; Zhang, Lee, & Marszalek, 2005). 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 in the slope of the force-extension curve. Despite this unique behavior, 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 bind-

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ing 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.

Consequently, polysaccharide stretching experiments to date have been largely dependent on picking up physisorbed polymers by chance from different surfaces, and attempting to locate favourable solution conditions for achieving the best stretches (Maurice & Matthai, 1999). In these circumstances definitive interpretation can be 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 indeed many studies report peeling of molecules from surfaces. However, occasionally a contact is made that is strong enough to suffice for a significant stretch to ensue, presumably because of some pinning induced by a surface feature of the substrate or by another overlapping chain. It follows that a large number of scans are needed in order to detect an effective stretch by such a methodology, involving tedious trials of several solvent conditions, making it particularly difficult to perform multiple scanning and stretch reversal studies.

If, alternatively, known points on the polysaccharide molecules, ideally the terminal residues, could be functionalized with handles to which the nano-scale force transducers (cantilevers or beads) could be attached, this would significantly improve the potential of this type of experiment. As mentioned, with nucleotides and proteins the abundance of functional groups with well-differentiated chemistry makes such an endeavor comparatively easy, while in sugars and carbohydrates, owing to the large number of functional –OH groups in the system, it becomes a complex procedure to perform chemistry at preferred positions. Nevertheless, herein, we have made an effort in this direction, attempting to convincingly attach one end of a polysaccharide molecule, pectin, to microbeads, and to characterize the specific covalent bond formed using spectroscopy supported by quantum chemical calculations.

The particular polysaccharide, pectin, was selected as: (i) the typical force-extension behavior of this important polysaccharide presents two force induced conformational transitions that make it a particularly interesting case (Williams, Marshall, Anjukandi, & Haverkamp, 2007): (ii) recent work on coupling low molecular weight pectic oligomers to substrates presented an opportunity to examine whether any of these reported couplings could be effectively used for polymeric compounds (Guillaumie, Thomas, & Jensen, 2002) and (iii) recent work carried out using ATR/FT-IR coupled with density functional theory (DFT) calculations for the analysis of pectin substrates seemed ripe for extension into the examination of coupling (Fellah, Anjukandi, Waterland, & Williams, 2009). For the purpose of this work pectin can be considered 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 postion (Ralet & Thibault, 2002). The previous study described the determination of the degree of methylesterification of pectins based on measurements of the ratio of the intensities of the CH_3 and backbone bands originating from linkages within the sugar rings. This permitted the elimination of the interferences from other cell wall components such as water and proteins that are found in a commonly used alternative method that employs monitoring the intensity of carboxylic bands (Gnanasambandam & Proctor, 2000; Haas & Jager, 1986; Monsoor, Kalapathy, & Proctor, 2001; Synytsya, Čopíková, Matějka, & Machovič, 2003). In order to validate proposed assignments, and confirm the frequencies associated with the new proposed methodology, IR spectra were calculated using DFT. Of late, there have been extensive studies of the IR and Raman properties of molecular systems using theoretical simulations (Krishnakumar, Keresztury, Sundius, & Ramasamy, 2004; Krishnakumar & Prabhavathi, 2008) and the revolutionary development in recent years in available computational facilities and large clusters have made the simulations of comparatively large systems, such as several sugar rings, possible (Fellah et al., 2009).

Herein, the use of this robust spectroscopy method supported by DFT calculations is investigated for its potential for validating specific bond formation in proposed coupling reactions involving pectin macromolecules and substrates, carried out in order to facilitate various single molecule biophysical experiments. While many indirect methods can assess the "sticking" of polymers to substrates, we aimed to investigate whether FTIR spectroscopy could allow the detection of specific newly formed bonds with surfaces. Such functionalization of different surfaces with pectin molecules could be utilised as the starting step for AFM and Optical Tweezers studies to obtain high-resolution structural and mechanical properties of these functionally important molecules.

2. Experimental

2.1. Materials

Pectin was obtained from Fluka. It had an anhydrogalacturonic acid content of 85%, a degree of methylesterification of 78% and a molecular weight of 30–100 kDa. All other chemicals were purchased from Acros Organics except $SnCl_2$ from Scharlau, Boc-Cys(trt)-OH from Bachem and hydrazine monohydrate from Alfa Aesar. Polystyrene beads (nominally 100 and 500 nm, 2.5% (w/v) solids aqueous suspension, Polysciences, USA) used in the experiments were first washed, removed from their respective solutions by centrifugation, and subsequently dried.

2.2. Coupling scheme

The immobilization of pectins onto pre-aminated beads was carried out via reductive amination (RA) or thiazolidine formation (TF). These methods have been successfully carried out for pectin oligomers (Guillaumie et al., 2002), but to our knowledge this is the first time they have been applied to polymeric molecules. Beads were selected as the substrate in preference to flat surfaces so as to increase the available surface area and thus the number of coupled species, in order to maximize the IR absorption. Polystryrene beads were selected as the major focus of the study (i) owing to their availability and common use in biophysical experiments and (ii) the complementarity of their IR spectra with that of pectin.

2.3. Polystyrene-bead amination

266 mg of polystyrene beads in a nitrating mixture of 0.532 mL of HNO_3 and 1.33 mL of H_2SO_4, were stirred at 60 °C for 30 min. The mixture was then poured over cold water in a refrigerated bath and washed several times with distilled water to remove excess acid. The polystyrene beads were then transferred to a mixture of 2.8 g of $SnCl_2$, 2.34 mL of HCl, and 2.6 mL of ethanol, and refluxed for 10 h at 90 °C. Upon completion of the reaction, the aminated polystyrene beads were washed with distilled water and 2 M NaOH, removed from solution by centrifugation and dried overnight under reduced pressure.

2.4. Immobilization of pectin

2.4.1. Reductive amination

Dried amino-terminated beads (50 mg) were washed with DMF. Succinic anhydride (320 mg, 3.2 mmol) and hydroxybenzotriazole (HOBt) (320 mg, 2.4 mmol) were dissolved in DMF (15 mL). N-N-Diisopropylethylamine (DIEA) (600 μL, 3.6 mmol) was added and the solution was added to the beads. The mixture was then

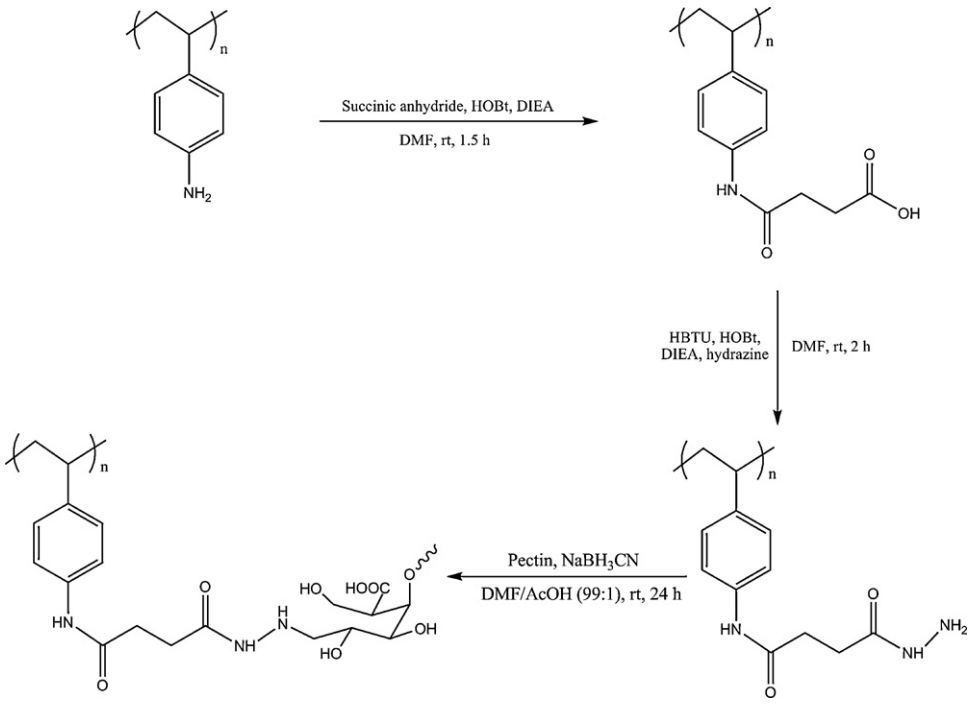

Fig. 1. A schematic of the reductive amination (RA) reaction scheme used for the attachment of the terminal sugar residue of pectin to aminated polystyrene.

stirred at room temperature for 1.5 h. Excess reagents and solvents were filtered off and the beads were washed with DMF, centrifuged and dried overnight under reduced pressure. The dried carboxylic acid-terminated beads synthesized were transferred and washed with DMF. O-Benzotriazole-NNN′N′tetramethyl-uronium-hexaflurophosphate (HBTU) (200 mg, 0.5 mmol) and HOBt (360 mg, 2.7 mmol) were dissolved in DMF (15 mL). DIEA (540 μL, 3.24 mmol) was then added, followed by hydrazine monohydrate (180 μL, 3.6 mmol) and the mixture was added to the beads. After 2 h coupling at room temperature, solvents and unreacted compounds were removed by filtration and the beads were washed with DMF, centrifuged and dried overnight under reduced pressure. Dried hydrazide-terminated beads were transferred and washed with DMF. Subsequently, pectin was dissolved in DMF-AcOH (acetic acid) (99:1), 0.5% (w/w); 3 mL of this solution was then added to the beads, and following addition of $NaBH_3CN$ (30 mg, 0.48 mmol), the mixture was then stirred at room temperature. After 24 h, the solvent and excess reagents were filltered off and the beads were washed with DMF, centrifuged and dried overnight under reduced pressure. The entire procedure is shown schematically in Fig. 1.

2.4.2. Thiazolidine formation

Dried amino-terminated beads (15 mg) were washed with DMF. Boc-Cys(trt)-OH (100 mg, 0.22 mmol) was coupled in the presence of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexaflurophosphate (PyBOP) (80 mg, 0.15 mmol), HOBt (15 mg, 0.12 mmol) and DIEA (30 μL, 0.18 mmol) in DMF (5 mL) at room temperature. After 1 h, the beads were washed with DMF, centrifuged and dried overnight under reduced pressure. Deprotection of Boc and trt groups was accomplished with TFA-CH_2Cl_2 (1:1) (5 mL) in the presence of Et_3SiH (50 μL, 0.3 mmol) for 1 h at room temperature. The beads were washed with CH_3CN, centrifuged and dried overnight under reduced pressure. Dried cys-terminated beads were transferred and washed with CH_3CN. Pectin was dissolved into CH_3CN–H_2O (1:2), 0.5% (w/w), and the solution, whose pH was decreased to 4 with 0.2 M HCl, was added to the beads. After 48 h at room temperature, the beads were washed with CH_3CN, centrifuged and dried overnight under reduced pressure. The entire procedure is shown schematically in Fig. 2.

2.5. Spectroscopy

Spectral acquisition was performed on solid samples using a Nicolet 5700 FT-IR spectrometer equipped with Omnic software (version 7.1) and a Smart Omni-Sampler (ATR cell with single reflectance germanium crystal). Each recorded spectrum is the average of 32 scans with a spectral resolution of 4 cm^{-1} from 400 to 4000 cm^{-1}, with a background spectrum recorded before each analysis.

2.6. DFT calculations

DFT calculations were investigated for their usefulness in predicting changes in the IR spectra resulting from the coupling of pectins to polystyrene beads. Molecular models for the pectin moiety immobilized on polystyrene beads (by reductive amination as well as thiazolidine formation) were built using Ghemical (Hassinen & Perakyla, 2001) and these were subsequently energy-minimized using the semi-empirical AM1 basis set. To make the system computationally less expensive, a pectinic acid dimer molecule attached to one unit of polystyrene was considered. The free valencies of the single styrene-monomer were satisfied by H atoms. Figs. 3 and 4 show the models used for the calculations of the vibrational spectra of the proposed intermediates and pectin-functionalized polystyrene beads respectively (a) during reductive amination and (b) during thizolidine formation.

DFT calculations were implemented in the *Gamess U S* package (Pople & Gordon, 1967). The computational facilities used consisted of an IBM-Bluegene cluster sited at the University of Canterbury, NZ. Bluegene/L nodes have two processors and two modes of execution are supported: co-processor (CO) and virtual node (VN) modes. In CO mode the first processor runs the program and the second processor handles I/O and communication on behalf of the first processor. In virtual node mode, both processors run the program. In CO mode all node memory is allocated to the first processor, while

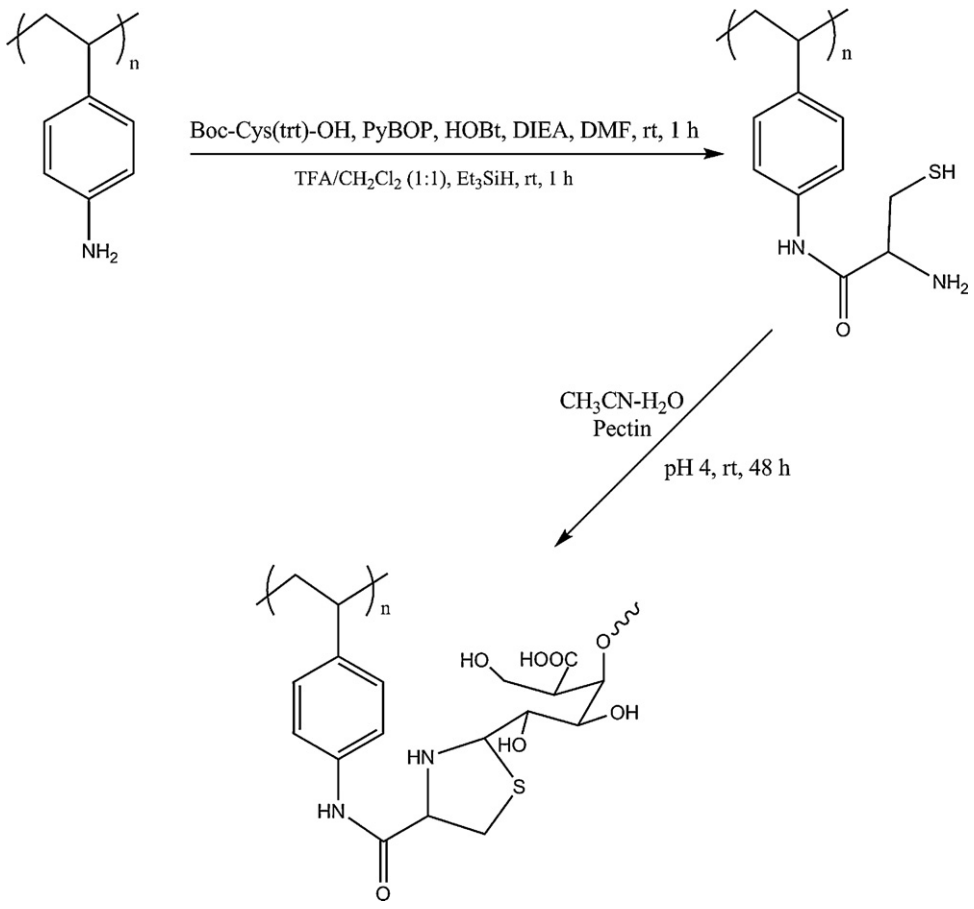

Fig. 2. A schematic of the thiazolidine formation (TF) reaction scheme used for the attachment of the terminal sugar residue of pectin to aminated polystyrene.

in the VN mode, the node memory is shared between the two processors. The single rack system utilised here provides a maximum of 1024 processors with 512 MB of RAM each, or 2048 processors with 256MB of RAM each; with the calculations reported herein exploiting the first option.

The structures of the reaction intermediates were first geometry optimized using the B3LYP/6-31G* basis and subsequently a complete optimization and Hessian calculation was undertaken using the B3LYP/6-311++G** basis set, incorporating the diffuse

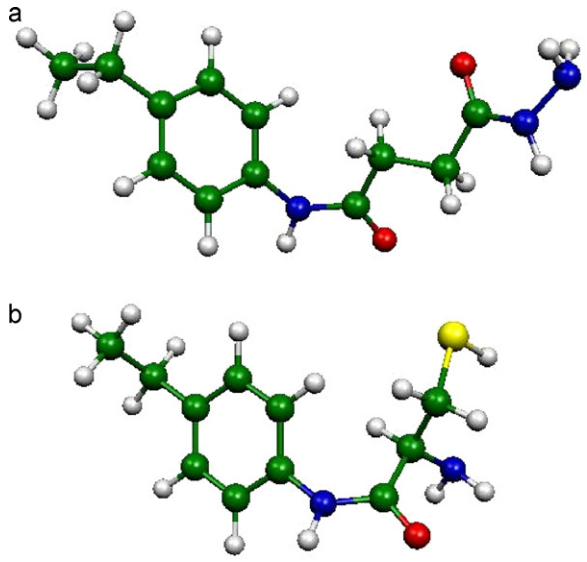

Fig. 3. Models for the intermediates formed (a) during the reductive amination pathway and (b) during thizolidine formation (carbon: green; oxygen: red; hydrogen: white; nitrogen: blue; suphur: yellow). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

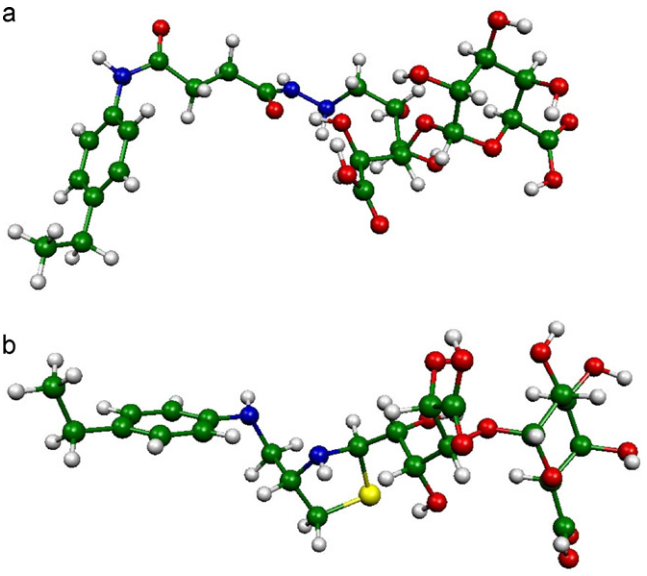

Fig. 4. Models of an immobilized pectin dimer coupled to a 'polystyrene bead' (a) by reductive amination and (b) via thizolidine formation, used in the DFT calculations of the vibrational spectra. (carbon: green; oxygen: red; hydrogen: white; nitrogen: blue; suphur: yellow). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

functionals. All simulations were performed at 0 K and in vacuum condition. The convergence criteria in the energy minimization for energy differences between cycles of optimization were less than 10^{-6} Hartree with the gradient set to be less than 10^{-4} a.u. The scaling and assignment of the vibrational modes were carried out using the Chemcraft program (Zhurko, 2009).

3. Results and discussion

Figs. 5 and 6 show the calculated IR spectra of galacturonic acid dimers (Fellah et al., 2009), bead (styrene-monomer) intermediates, and galacturonic acid dimers covalently coupled onto the polystyrene beads intermediates via the two methods discussed above. The (green) dotted lines indicate spectral features clearly associated with pectin while the (blue) dashed lines indicate those originating from the bead intermediate. The origin of these features is reported in Tables 1 and 2. While the intensities of the simulated bands should not be taken literally as predictions of the strengths of experimental features the calculated spectra can be interrogated in order to locate the relative frequencies of distinct vibrational

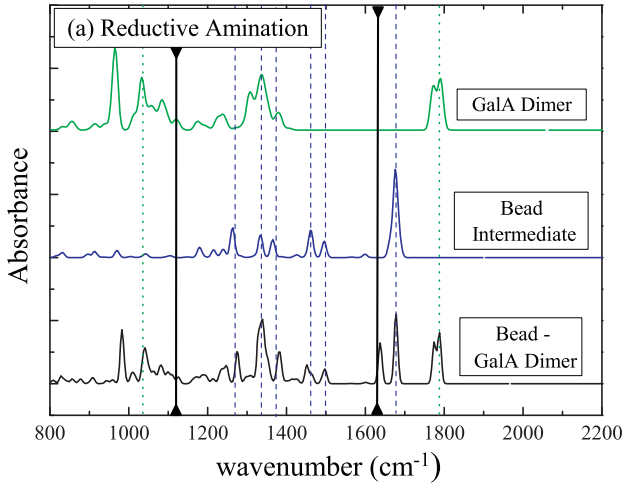

Fig. 5. The simulated IR spectra for a pectin model (galacturonic acid dimer) immobilized on a polystyrene bead model (functionalized styrene monomer) by the reductive amination method described herein. (For molecular models see Figs. 3 and 4(a).)

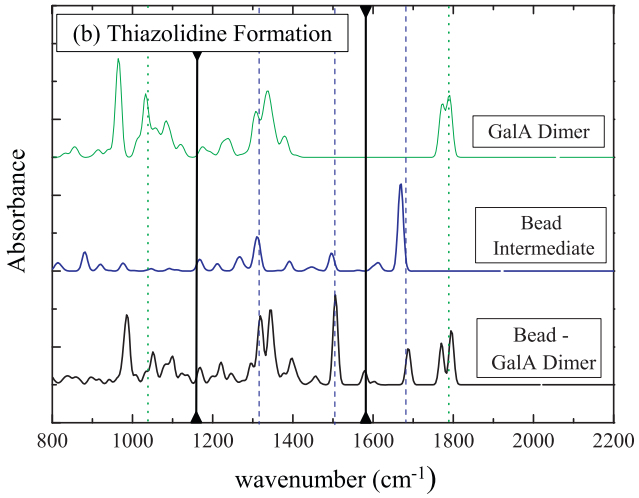

Fig. 6. The simulated IR spectra for a pectin model (galacturonic acid dimer) immobilized on a polystyrene bead model (functionalized styrene monomer) by the thizolidine formation method described herein. (For molecular models see Figs. 3 and 4(b)).

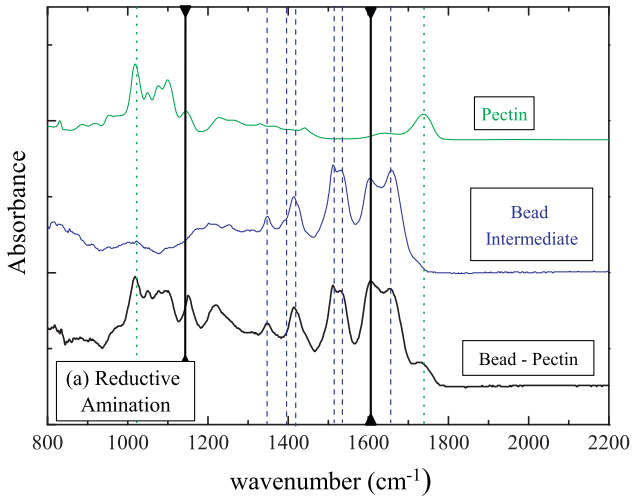

Fig. 7. Experimental IR spectra measured for pectin, and pectin immobilized on polystyrene beads using reductive amination, compared with the functionalized inter-mediate beads. The black solid lines indicate the positions at which the DFT calculations indicate vibrations relating to the newly formed covalent coupling are expected to occur.

features associated with the specific bonds proposed to have been introduced in the trialed coupling schemes. The simulated spectra of the coupled systems therefore highlight the potential locations of distinct peaks associated with any newly formed bonds after pectin immobilization in the experimental spectra. For example, it can clearly be seen that beads successfully attached to the end residue of the pectin chain bead are predicted to exhibit a newly formed C-N bond.

Figs. 7 and 8 show the measured experimental IR spectra of (a) pectin, (b) described bead-intermediates directly prior to pectin attachment, and (c) the beads with putative pectin coupling, via the reductive amination and thiazolidine formation, respectively. Once again (green) dotted lines indicate spectral features clearly associated with pectin while the (blue) dashed lines show vibrational frequencies originating from features of the bead intermediates. The assignment of these bands is detailed in Tables 1 and 2 (Appell, Strati, Willett, & Momany, 2004; Krishnakumar & Prabhavathi, 2008; Krishnakumar et al., 2004;

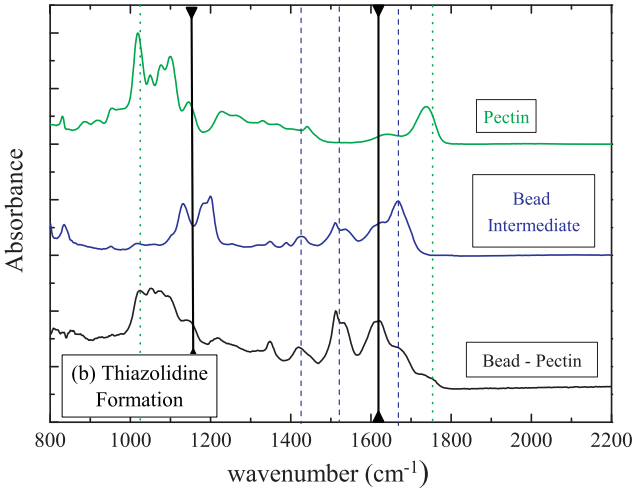

Fig. 8. Experimental IR spectra measured for pectin, and pectin immobilized on polystyrene beads using thizolidine formation, compared with the functionalized intermediate beads. The black solid lines indicate the positions at which the DFT calculations indicate vibrations relating to the newly formed covalent coupling are expected to occur.

Table 1
The assignment of relevant IR peaks for the reductive amination reaction and the comparison of the experimentally measured frequencies with those found directly from DFT calculation expounded herein (PIB: pectin immobilized on beads, AB: aminated intermediate beads, PGA: polygalacturonic acid).

PIB Cal: (cm^{-1})	PIB Exp: (cm^{-1})	AB Cal: (cm^{-1})	AB Exp:: (cm^{-1})	Assignment
1795	1720	–	–	υ(–C═O), PGA
1720	1660	1735	1660	υ(–C═O), bead
1650	1520	1635	1520	υ, benzene ring
1230	1210	–	–	υ(–CO), υ(–CC), pectin ring
1125	1145	–	–	υ(–CN), new covalent coupling
1610	1660	–	–	υ(–C≈N), new covalent coupling
1040	1010	–	–	υ(–CO), υ(–CC), δ(OCH) Ring

Table 2
The assignment of relevant IR peaks for thizolidine immobilization and the comparison of the experimentally measured frequencies with those found directly from DFT calculation expounded herein (PIB: pectin immobilized on beads, AB: aminated intermediate beads, PGA: polygalacturonic acid).

PIB Cal: (cm^{-1})	PIB Exp: (cm^{-1})	AB Cal: (cm^{-1})	AB Exp:: (cm^{-1})	Assignment
1815	1760	–	–	υ(–C═O), PGA
1720	1680	1720	1660	υ(–C═O), bead
1620	1550	1620	1520	υ, benzene ring
1267	1210	–	–	υ(–CO), υ(–CC), pectin ring
1202	1210	1205	1215	υ(–CN), bead
1125	1145	–	–	υ(–CN), new covalent coupling
1590	1610	–	–	υ(–C≈N), new covalent coupling
1040	1010	–	–	υ(–CO), υ(–CC), δ(OCH) Ring

Momany & Willett, 2000; Momany, Appell, Strati, & Willett, 2004; Venyaminov & Kalnin, 1990).

While the presence of pectin on the bead substrates following the described procedures could be inferred from washing assays and the results of the light scattering experiments, the goal of the spectroscopy experiments was the detection of the specific covalent bond formed in coupling and hence confirmation that the desired coupling chemistries had been successful and that specifically the terminal sugar residue was attached (thereby potentially maximizing the length of polymer stretched in single molecule experiments). As mentioned both coupling modalities were expected to involve the formation of a new –C–N bond after the pectin immobilization that potentially could be monitored in the IR spectrum. In the case of the reductive amination technique, this new pectin-substrate bond is unique as it is the only –C–N bond in the system while there is a pre-existing –C–N bond in the thiazolidine coupled system intermediate prior to pectin attachment that has to be considered. Such C–N bonds are expected to show clear signatures at around 1120–1150 cm^{-1} and additional vibrations around 1580–1670 cm^{-1} that reflect the delocalised character of the –C–N; being coupled with –C═O in the reductive amination case and strained in the five-membered ring formed in the thizolidine case. The black lines in the experimental figures indicate the position of the predicted signature vibrations arising from the new bond formed on coupling and there can indeed be seen to be extra intensity at these frequencies in the experimental data, consistent with coupling the polysaccharide moiety via newly formed chain-end covalent bonds. This is particularly clear for the RA case, while with TF it is less clear-cut with the new-bond intensity predominantly broadening pre-existing peaks.

Despite the fact that, owing to the crystalline, single-unit nature of the substrate in the simulations, peaks in the simulated spectra are sharp compared with typical experimental bands, the coincidence of the calculated end-residue-covalent-coupling bands, indicated by the (black) solid lines, with extra experimentally detected intensity is strong evidence supporting the proposed nature and position of the attachment. It should also to be noted that the simulations correspond to vacuum conditions and 0 K, unlike the experiments that are performed at the room temperature, and the absolute values of the calculated frequencies do typically deviate slightly from the experimental ones by up to 50 wave numbers. It is routine to scale the frequency axis by a small amount so as to align major bands with those experimentally observed and the co-alignment of multiple calculated and experimental bands via the same scaling, as seen here, is typically taken to constitute good agreement.

Other prominent bond vibrations seen in the experimental IR spectra, as detailed in Tables 1 and 2, are those corresponding to a set of carbonyl (–C═O) groups in the system at around 1650–1750 cm^{-1} and the benzene ring vibrations at around 1550 cm^{-1}. In the case of pectin and pectin-functionalized beads, the pectin backbone vibrations can be seen at 1000–1100 cm^{-1}. The addition of a new shoulder peak in the IR spectra, owing to the –C═O stretching of the pectin entity at around 1715 cm^{-1} also adds support to the attachment of the pectin molecules on the beads (although not to the nature of the bond). Once again similar behavior was found in the simulated IR spectra.

4. Conclusion

It was recently suggested that vibrational spectroscopy coupled with quantum chemical calculations might be a useful tool to examine the success of particular coupling schemes designed for the attachment of biopolymers to substrates as part of an effort to facilitate modern biophysical experiments. It has been shown herein that indeed FT-IR results, when considered with predictions from DFT calculations, can provide strong evidence for the success of specific coupling schemes. A good general agreement between the experimental and simulated spectra pre-, as well as post-, coupling confirm the reliability of this technique. In particular, this work provides evidence that by following the protocols described not only can the pectin chain be attached to microbeads, but that the coupling is via the formation of a specific recognizable C–N bond, with pectin coupled at its reducing end. It is hoped that the availability of such a technique will encourage further work on specific coupling and will ultimately facilitate more sophisticated biophysical experiments to be carried out on polysaccharides and their interactions.

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