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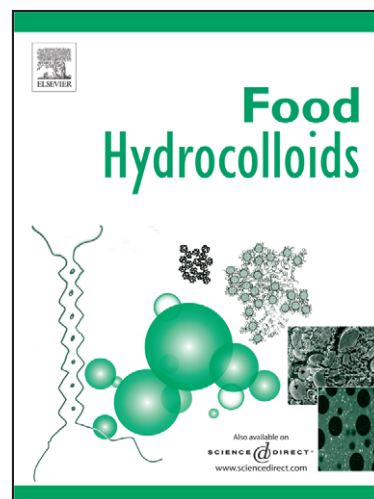
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**Stretching single polysaccharide molecules using AFM: A potential method for
the investigation of the intermolecular uronate distribution of alginate?**

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Abstract

Illustrative examples of the way in which the molecular force-extension behaviour of polysaccharides is governed by the nature of the linkage between their constituent pyranose rings are presented for a series of standard homopolymers. These results agree with previously proposed general hypotheses regarding the possibility of generating force-induced conformational transitions, and with the predictions of a model in which the inter-conversion of pyranose conformers is assumed to be an equilibrium process on the timescale of the molecular stretching. Subsequently, we investigate the potential of the technique in the characterisation of co-polymeric polysaccharides in which the nature of the glycan linkages is different between the two distinct residue types. Specifically, we explore the possibility that the ratio of

mannuronic acid (M) to guluronic acid (G) in alginate chains will be reflected in their single molecule stretching behaviour, owing to their contrasting equatorial and axial linkages. Furthermore, as the technique described interrogates the sample one polymer at a time we outline the promise of, and the obstacles to, obtaining a new level of characterisation using this methodology where differences observed in the single molecule stretching curves obtained from single alginate samples reflect something of the real intermolecular distribution of the M / G ratio.

Keywords: AFM, Single molecule stretching, Polysaccharides, Nano-mechanics, Alginate, Force-induced conformational transitions.

Introduction

Atomic force microscopy (AFM) has emerged as the technique of choice for conducting single molecule force spectroscopy, owing largely to the limited range of forces that can be applied by competing methodologies such as the use of optical tweezers or magnetic beads (Strick et al., 2003). Single polynucleotides, proteins, and synthetic polymers have all been stretched using AFM (Oberhauser, Marszalek, Erickson & Fernandez, 1998; Ortiz & Hadziioannou, 1999; Strick et al., 2003; Harris, 2004.) and in addition an increasing number of studies are being carried out on polysaccharide molecules (Zhang, Lee & Marszalek, 2005, Haverkamp, Williams and Scott, 2005; Zhang & Marszalek, 2006; Walther, Brujic, Li. & Fernandez, 2006). At first sight polysaccharides may appear not to offer the same exciting opportunities as nucleotides, whose duplexes can be mechanically separated, or proteins, that may be mechanically denatured. They do, however, exhibit their own richness of behaviour including, uniquely among the biopolymers, the possibility of force-induced

conformational transitions of the pyranose rings (Marszalek, Oberhauser, Pang & Fernandez, 1998; Marszalek, Pang, Li, El Yazal, Oberhauser & Fernandez, 1999).

The fact that polysaccharides are key elements of many biomaterials, including plant cell walls, has fuelled increasing speculation about the potential physiological significance of such force-induced transitions. It has been suggested that they may have a signalling role *in-vivo* (Marszalek et al., 1999), and indeed their presence is likely to provide a way of moderating the interaction of polysaccharides with a variety of other molecules, either in signalling pathways or in the control of intermolecular interactions of direct mechanical relevance, such as those described recently in bone glue, where sacrificial polymer associations provide a mechanism for dissipating energy (Fantner et al., 2005). Furthermore, recent work (Haverkamp et al., 2005) has demonstrated that these force-induced conformational changes occur in the sugar rings of one family of bridging anionic glycosaminoglycans (AGAG), the dermochondans, but not in the closely related chondroitans. As tissues requiring significant elastic extensions (e.g. skin) characteristically contain dermochondans rather than chondroitans the work suggests that the nanomechanics of force-induced conformational transitions of pyranose rings may play a role in directly controlling macroscopic tissue properties. Understanding the generation and utilisation of such function might ultimately allow polysaccharides with designed architectures to be utilised in a host of biomimetic soft nanotechnology applications including sensors and advanced materials.

Conversely, by using measured single polysaccharide force-extension behaviour as a signature of biopolymer architecture, such methods hold the promise of polysaccharide sample characterisation at a previously unprecedented level of molecular detail. In this paper we first present illustrative examples of how

polysaccharide structure, in particular the nature of the linkage between pyranose rings, impacts on molecular force-extension behaviour. Subsequently, having illuminated the structure-function relationship between the presence of features in the molecular stretching curve and the conformation of constituent linkages, we investigate the potential of the technique in the characterisation of a co-polymeric polysaccharide, in which the nature of the glycan linkages is different for distinct residues types.

Current State-of-the-Art in Polysaccharide Stretching

Single-chain stretching data obtained from experiments carried out on polysaccharides that do not contain force-induced conformational transitions are routinely fitted to statistical mechanical models of the force-extension curve, in particular the freely jointed and wormlike chain models (FJC and WLC models) (Strick et al., 2003). Extensions to these models, which involve an extensible high-force Hookean regime that permits the chain to be elongated beyond its contour length (Janshoff, Neitzert, Oberdorfer & Fuchs, 2000) have also proved useful and allowed the extraction of parameters relating to the elasticity of the straightened polymer. In general these models show excellent utility in describing experimentally measured curves although the extracted values of persistence or Kuhn lengths (WLC and FJC respectively) from such fits are questionable in their physical significance, often giving values of less than one sugar ring in length.

Much recent theoretical work has concentrated on the possibility of disentangling volume interactions or even sequence information from heteropolymers (Etchegoin & Maher, 2003; Jarkova, Lee, & Obukhov, 2005), while in the experimental arena variations on the AFM stretching technique such as using a force-ramp (Marszalek,

Li, Oberhauser, & Fernandez, 2002) or dynamic measurements (Humphris, Antognozzi, McMaster & Miles, 2002) are being developed, and issues around functionalising AFM tips investigated (Friedsam, Bécares, Jonas, Gaub, & Seitz, 2004). Additionally, recent work has used the resonant modes of the AFM cantilever to investigate polysaccharide transitions under rapid stretching conditions (Walther et al., 2006), and the dependence of the observed behaviour on solvent conditions has also been studied (Zhang & Marszalek, 2006).

Rules regarding whether particular polysaccharides exhibit force-induced conformational transitions have been proposed based on the linkage patterns of the sugar rings (Marszalek et al., 1999; Marszalek, Li, & Fernandez, 2001). Equatorial-linked polysaccharides such as cellulose already exist in their most extended conformation and the sugar ring cannot deform under tension. However, in polysaccharides in which the pyranose rings are axially-linked the bond can act as a molecular lever inducing conformational changes in the sugar rings under tension. Hence, pyranose based polymers with a 1-4 glycosidic linkage with one axial and one equatorial bond, such as amylose (1a-4e), can undergo one main conformational transformation under tension. Those with a 1-4 linkage but with both bonds axial, such as pectin (1a-4a), undergo at least two conformational transformations. Intermediate states may also exist such as a skewed boat form. Herein we refer to all these force-induced transformations from a shorter to a longer conformation as “clicks”.

The force-extension curves of such systems have been modelled theoretically as elastically coupled two-level systems using Monte-Carlo simulations (Rief, Fernandez, & Gaub, 1998) and the extension by AFM of clicking polysaccharides was found to be an equilibrium process, except at very high pulling rates, in contrast to

proteins, that are more commonly in the non-equilibrium region. An expression that exploits the equilibrium nature of the transition has recently been developed in order to describe the physical behaviour of a polysaccharide molecule as it is stretched from the entropic region, through one or more ring conformational transformations, into the Hookean regime (Haverkamp, Marshall & Williams, 2006). This model adapts existing models in order to accommodate one or more force induced conformational transformations of the glycan rings and is based on the concept of equilibrium between the clicked (longer conformers) and unclicked states. The equilibrium is determined by the Gibbs energy difference between the states and is perturbed in favour of the clicked states by the force applied to the molecule. In addition, ab-initio calculations have been used to investigate the nature of the actual conformations involved (O'Donoghue & Luthey-Schulten, 2000; Lee, Nowak, Jaroniec, Zhang & Marszalek, 2004). While a number of different polysaccharides have been stretched and the linkage hypothesis tested extensively, the vast majority of studies have been on homopolymers.

Polysaccharide fine structure

Polysaccharide samples are often highly heterogeneous and in general contain structural variation both within and between individual chains. The reason for this is in part that they are secondary gene products and rely on a spatially and temporally concerted conspiracy between several proteins for their biosynthesis, rather than being directly templated. Perhaps more importantly, particularly with structural polysaccharides, is that it is their ability to be post-depositionally modified that is key to their functionality. That is; base structures are often generated, incorporated into biomaterials and are subsequently, according to their position and the state of a

myriad of feedback systems, modified by a host of modifying enzymes in order to maximise their function.

Polysaccharide co-polymers and alginate

The simple control of the pattern of two constituent residues in linear co-polymeric structures is sufficient to open up a huge range of possibilities regarding the potential association of molecules. For example, witness the different properties of pectins, alginates and galactomannans possessing blockwise distributions of their constituent residues with respect to analogues in which the relevant co-polymeric constituents (galacturonic acid and its methylesterified version, mannuronic(M) and guluronic(G) acid, and naked and galactose-substituted mannose, respectively) are distributed randomly. Unfortunately, in tandem with the introduction of flexibility of function, such variations also increase the complexity of the characterisation of a polysaccharide sample enormously, with inter- and intra-molecular distributions of a particular residue type to be considered. While of course for some manifest physical properties, such as the viscosity of a dilute solution, only limited information regarding the chain structure might be required, there is, as implied above, ample evidence to show that such distributions are relevant for intermolecular associations of any kind; including those with a mechanical function. Equally, it is likely that binding epitopes relevant in signalling processes will be based on exquisitely tuned patterns of recognition.

In the case of pectin the constituents of the co-polymer are distinct simply owing to methylesterification so that intermolecular distributions of the degree of methylesterification are reflected in the presence of chains within the sample

possessing different overall charges. A measure of the intermolecular distribution of methylesterification can therefore be obtained simply by distinguishing between chains electrophoretically. In alginate, however, both residues possess similar charge so that determining their chain-wise variation is not so facile.

A schematic of alginate fine structure is shown in figure 1 and while the pKa's of mannuronic and guluronic acid may be difficult to distinguish, it is clear that they exhibit structural differences, in particular a C5 epimerisation and a different linkage. Some information regarding the sample averaged relative triad frequencies (the occurrence of MMM, GGG etc) can be obtained from NMR (Grasdalen, Larsen & Smidsrod, 1979; Stokke, Smidsrod, Bruheim, Skjakbraek, 1991), that give some information regarding intramolecular patterns of residue placement but any attempt to reconstruct an average chain based on this information must assume something about the intermolecular distribution. Complementary information on intra-molecular patterns might be obtained from digests with alginate enzymes (Ostgaard, Stokke & Larsen, 1994) (the same is true for pectin), but there is little, other than perhaps the lack of digestible motifs, that can be said about whether each chain possesses the quoted M / G ratio; or whether a large range is present.

Here we explore the possibility that the M / G ratio will be reflected in their single molecule stretching behaviour, and therefore aim to investigate the potential of AFM single molecule stretching for obtaining information on its intermolecular distribution.

Materials and Methods

Polymers.

Methylcellulose (Mw 86 kDa) and carboxymethylamylose were purchased from Sigma and used without further purification. Alginates (Mw 150 kDa) were kindly supplied by FMC Biopolymers and had sample average guluronic acid contents of 30 and 70 %, as determined by NMR.

AFM.

Force-distance curves were recorded by pulling polysaccharide molecules at 500 nm s^{-1} using a scanning probe microscope (Veeco Nanoscope E) with a Si AFM tip (Ultrasharp CSG11, NT MDT Co, Moscow, Russia, tip radius ca. 10nm). The samples was prepared by applying 50 μl of 0.01% solutions in H_2O to a clean glass disc, which was then dried under vacuum before mounting in the AFM and filling the cell with water just prior to the force curve measurements.

Results and Discussion

Figure 2(a) shows the recorded single molecule force versus extension curve for methylcellulose. The extension is normalised by the contour length and the observation that normalised stretches superimpose is taken as good evidence for single molecule events. Furthermore, it can be seen that the extensible wormlike chain describes the data well, the fit yielding a specific modulus of $(4900 \pm 500) \text{ pN}$, in good agreement with previous work (Abu-Lail & Camesano, 2003). As expected from its (1e-1e) linkage configuration, no clicks are observed.

In contrast, data obtained from the (1a-1e) carboxylmethylamylose clearly shows a click, as predicted (figure 2(b)). Excellent agreement is found to a recently proposed model based on a WLC description with a variable contour length, given by the current equilibrium state of clicked and unclicked residues (Haverkamp, Marshall &

Williams, 2006). The extracted value of Gibbs energy between the longer and shorter conformers is found to be, $\Delta G_0 = 18 \text{ kJ mol}^{-1}$ in line with 12-20 kJ mol^{-1} quoted by other studies (Kildeby, Melberg, & Rasmussen, 1977; Joshi, & Rao, 1979).

Finally, results from the literature (Marszalek et al, 1999) for (1a-1a) pectin are again in line with the basic structure-function hypothesis and two clicks are now discernable (figure 2(c)). Once again a WLC model, modified to account for the presence of clicks, describes the data well.

These plots graphically illustrate the proposed structure-function relationship between the presence of clicks and the nature of the pyranose linkages. Furthermore, they give confidence in our ability to model the force extension curve of a polysaccharide given the linkage pattern, the relevant Gibbs energy and length of any conformers (Haverkamp, Marshall & Williams, 2006). Although the data shown in figure 2 is for homopolymers, it is a trivial exercise within the framework of the model to extend the predictions to the case of chains containing differentially linked residues. Armed with our predictive model, and a single chain view of the sample, we now turn our attention to the stretching behaviour of alginate samples characterised by varying M/G ratios.

We first assumed a set of reasonable parameters for the alginate chain (ring lengths representative of the stable conformers of 0.435, 0.4833 and 0.5175 nm (Atkins, Mackie, Parker & Smolko, 1971), Gibbs energy differences of 11.6 and 20.6 kJ mol^{-1} and a persistence length and specific modulus for the chains of 1 nm and 60 nN respectively (Abu-Lail & Camesano, 2003). Using this data we calculated the force-extension curve for a variety of guluronate compositions, in order to assess how

experimentally distinguishable we would expect the behaviour of chains with varying M / G ratios to be. It is assumed that the persistence lengths and specific stiffnesses of the chains will also be a function of guluronate content, but preliminary calculations showed that the magnitude of expected changes are unlikely to affect the main conclusions presented here, and they have therefore been kept constant for the sake of simplicity. The data obtained for each composition has been normalised to the polymannuronate simulation at a force of 2 nN. It is striking that there is a clear difference in the functional form of the stretch as the guluronate content is varied that suggests our approach may well be a promising one (figure 3). It should be noted that while the model predicts the number of glycan rings in each conformer as a function of force, it presently lacks an interaction term. As such the predicted form of the curve is sensitive only to the total amount of clickable rings within a stretched chain and not their intramolecular pattern.

Indeed, it is immediately clear from our experimental data that there is a substantial difference between the response of chains from the different M / G samples and furthermore that the trend agrees well with that predicted (figure 4). While the calculations suggest that differences of around 10 % in the M/G ratio might be discernable from the form of the force-extension curve the magnitude of the noise in these experimentally determined curves is too large at present to permit reliable fitting. It can be seen, however, that there is some variation in the exact form of the click especially for one of the 70% M / G stretches. We suggest that this variation arises from the fact that we are in fact sampling the intermolecular M / G distribution of the samples. However, it is important to appreciate that it is also possible that the intra-molecular distribution of residues could also play a role in this regard. Typically

in the measured curves we pick up portions of the polymeric chains with lengths of the order of around 100 nm, while the molar mass information we have suggests that the average chain length is some 750 sugar residues. This means that if a chain has a blocky intramolecular distribution of co-polymeric residues it is possible that the M / G ratio reflected in the molecular stretch may be different depending simply on which section of a particular chain is stretched. While this holds some promise for the possible monitoring of the intramolecular distribution by this technique it further complicates the interpretation of the data. In addition, the sampling of the chains cannot straightforwardly be assumed to be random. It is quite possible, owing to subtle changes in the chemical nature of the residues and the local geometry of the chain, that certain chains (of particular M / G ratios) and indeed certain parts of certain chains (with particular M / G patterns) will preferentially interact with the surface of the substrate or the AFM tip and be over or under-represented in the force-curves measured from a sample. Indeed, previous work has suggested using force-measurements as a fingerprinting methodology, for characterising mixtures of homopolymers (Marszalek et al., 2001). While this work showed that force measurements could be used to recognise the presence of homopolymers with one clicking pattern when mixed with another exhibiting a different response, quantification of the different polymers from counting the number of each type of stretch was not possible. Indeed, the experimental ratio determined in this fashion was found to be highly dependent on the nature of the substrate surface. It is clear that these considerations could have implications for the work described here and a better understanding of the polymer – substrate interaction in stretching experiments will be required to ameliorate the situation.

Conclusion and Outlook

Qualitatively we have shown that the force-extension curves of single polysaccharide co-polymers reflect the linkages within the stretched section of chain. These results agree with the previous proposed general hypothesis regarding the ability of certain conformations to facilitate clicks, and with the predictions of a model in which inter-conversion of pyranose conformers is an equilibrium process on the timescale of the molecular stretching. The success of our model in reproducing the force-extension curves of a representative set of homopolymers gives us confidence in its predictive capability and indeed we have calculated a trend for alginate samples of differing guluronate contents that is broadly matched by experiment. In addition we have observed from the single molecule stretching an indication of chains with different guluronate contents being present in a single alginate samples. We firmly believe that future progress will make robust characterisation of the intermolecular distribution of guluronate residues using this technique a reality. However, at present, there are a number of currently unresolved issues that will require addressing before such intermolecular distributions can be confidently asserted. These largely resolve around gaining a better understanding of the attachment of the polymer to the tip and substrate so that it is known how preferential interactions of particular co-polymeric motifs might influence the sampling of the chain structures, and discerning the influence of the attachment geometry.

Future work will involve obtaining more extensive statistics on the different stretches obtained from single samples under different solution and substrate conditions, coupled with modelling of the M / G distributions using information from other sources, and investigating possible chemical attachments with a view to circumventing some of the problems described.

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Figure Captions

Figure 1: A schematic diagram of the fine structure of alginate showing (a) the constituent sugars, (b) their relevant linkages, and (c) possible intra-molecular patterns of the different sugars.

Figure 2: Force extension curves of (a) methylcellulose, (b) carboxymethylcellulose, and (c) pectin data taken from Marszalek (Marszalek et al, 1999). Open circles are experimental data and the solid lines are fits to models as described in the text.

Figure 3: Simulated force-extension curves for alginates of varying guluronate content.

Figure 4: Representative experimentally determined force-extension curves recorded on samples with average guluronate contents of 70% and 30%.

Figures

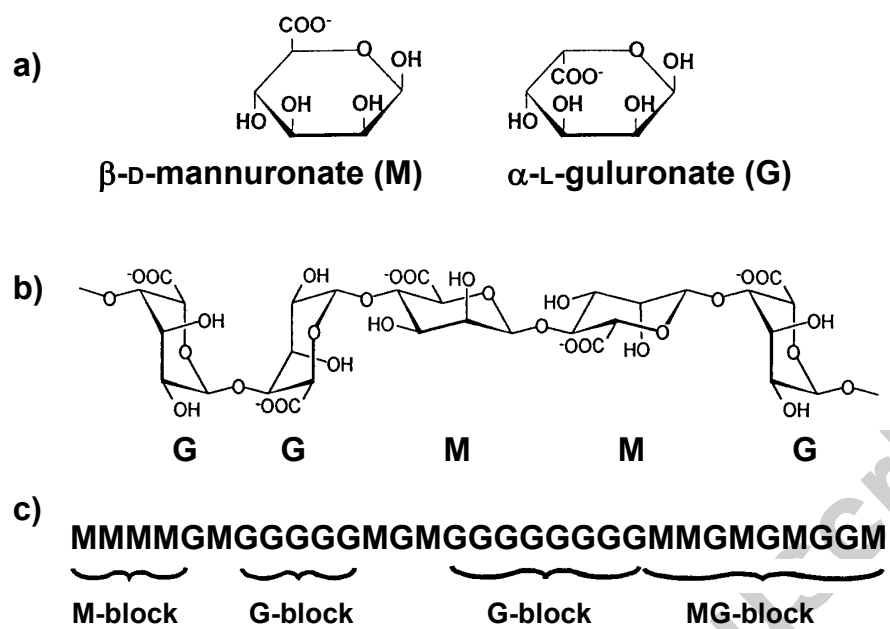


Figure 1

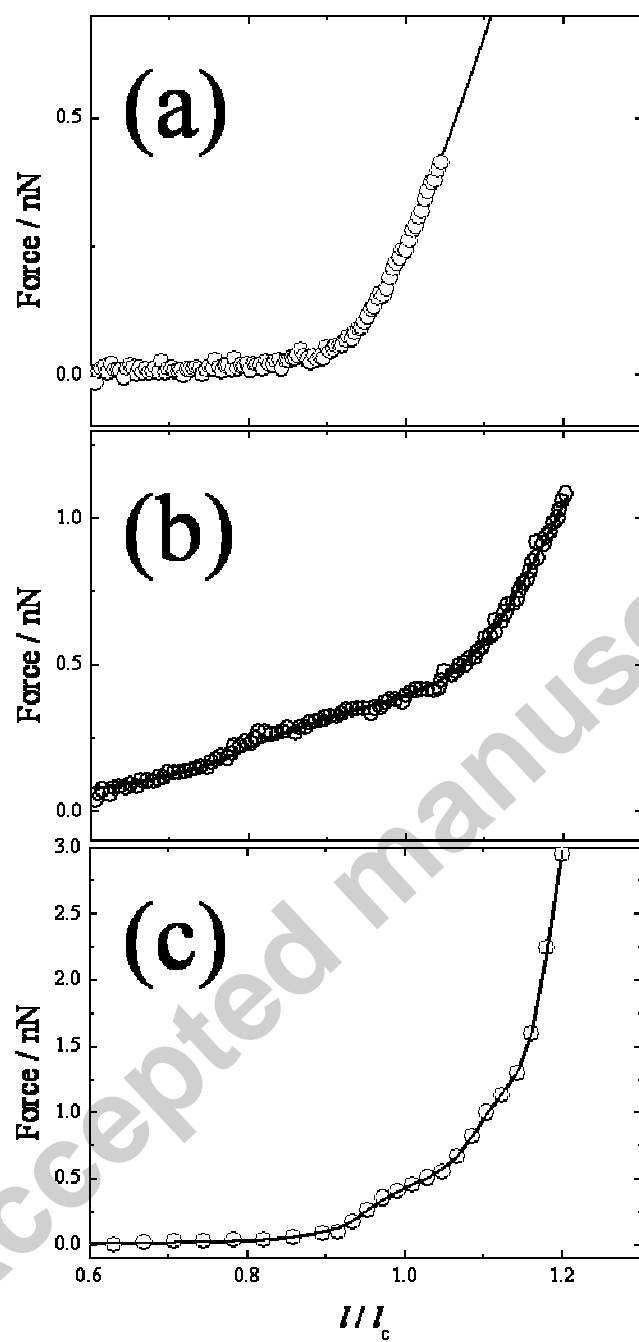


Figure 2

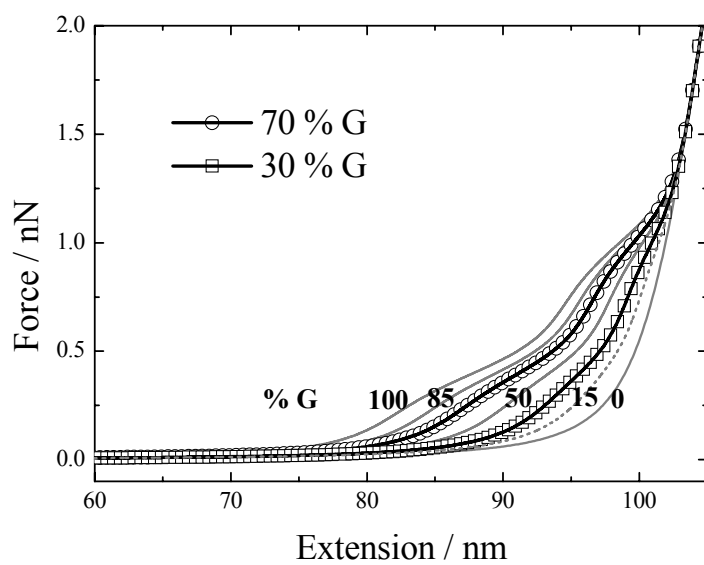
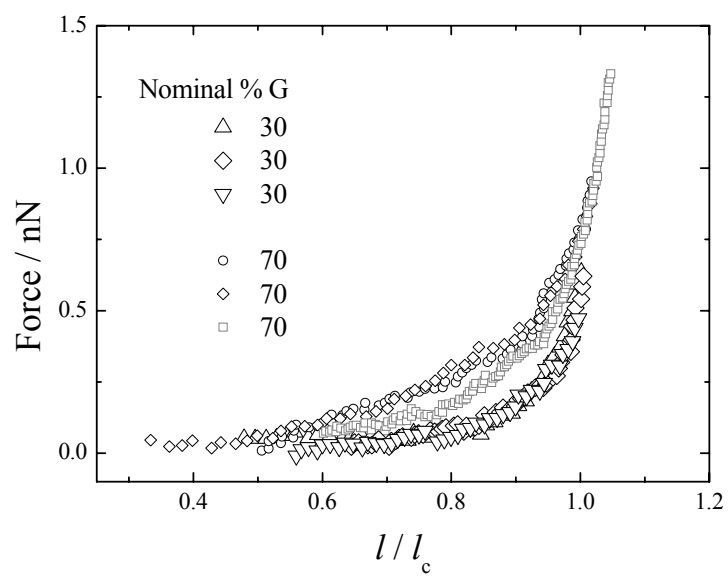


Figure 3

**Figure 4**

Stretching single polysaccharide molecules using AFM: A potential method for the investigation of the intermolecular uronate distribution of alginate?

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