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Imaging bacterial polysaccharides by AFM

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Summary
Atomic force microscopy has been used to image the bacterial polysaccharides xanthan, acetan and gellan. Images were obtained under constant force conditions in a liquid cell. Drops of dilute solutions of the polysaccharides were deposited onto freshly cleaved surfaces of mica and allowed to dry in air. The deposits were then imaged under butanol. Xanthan and acetan form entangled networks upon deposition. Individual molecules can be identified. Under suitable circumstances it has been possible to image the helical structure of acetan. Gellan forms gels upon concentration during drying and images have been obtained of the gel network.

Introduction
Scanning probe microscopies, and in particular atomic force microscopy (AFM), are new techniques for studying polymers (1). This article will be concerned with the use of AFM to image polysaccharides. Types of polysaccharide structure range from simple polymers with regular chemical repeat units which adopt well defined secondary (helical) structures, through those containing minor irregularities in chemical structure, to more complex block copolymers, and finally very complex irregular or branched structures. This article describes studies using bacterial polysaccharides as models for probing the ability of AFM to image general polysaccharide architecture. The three polysaccharides chosen (xanthan, acetan and gellan) all possess regular chemical repeat units (2-4) and adopt known helical structures (5-7). Gellan is an extremely effective gelling agent (8) offering the possibility for probing the network structure formed upon intermolecular association.

There are at present only a few reports concerning scanning probe microscopy of polysaccharides (9-21). Most of these studies (9-17) have been made by scanning tunnelling microscopy (STM). Images are clearly difficult to obtain. The quality of the STM images obtained has been disappointing, and most of the studies have not been reproduced by other workers. A major problem appears to be that the STM probe damages or displaces the macromolecules. Polysaccharides will then only be imaged when they become trapped at defects on the substrate making the imaging process irreproducible. This difficulty can be avoided if the molecules can be organised into arrays which resist displacement (9,15) or otherwise immobilised on the substrate (16, 17). Three groups have reported STM images of the bacterial polysaccharide xanthan (14-17). Miles and coworkers (14) present images of individual xanthan molecules air dried onto graphite. Although the images show features consistent with the xanthan structure it has not been possible to reproduce these images using the same preparative procedure (15-17). Gunning and coworkers (15) imaged xanthan by air drying aligned liquid crystalline preparations onto graphite. Wilkins and coworkers (16,17) immobilised xanthan molecules by metal coating air dried deposits

on graphite or mica substrates. As will be shown in this article, although the latter method permits reliable imaging of polysaccharides, the grain size of the metal coating restricts the potential resolution achievable with scanning probe microscopy.

Atomic force microscopy (AFM) offers advantages over the use of STM. It is possible to control the imaging force and thus minimise molecular distortion and displacement. In addition a wider range of substrates can be used for imaging macromolecules. Hanley and coworkers have reported (18) AFM studies of cellulose microfibrils and claim to have observed a periodicity associated with the cellulose helix at the surface of the crystallites. Meyer and coworkers (19) report preliminary studies of xanthan adsorbed to mica, although the images presented show continuous periodic arrays with no visible polymer ends. Recently a method has been described (20,21) which allows reproducible imaging of polysaccharides using AFM. Data presented in the present article confirms the use of this method for imaging uncoated polysaccharides. Comparative studies of coated and uncoated polysaccharides demonstrate the loss of resolution caused by coating the samples. Finally the method has been applied to image the network structure of a gellan gel.

Experimental

Samples of xanthan (Keltrol) and gellan (Gelrite) were purchased from Kelco-AIL. Xanthan is a mixed salt form of the polysaccharide. Gelrite is the de-esterified product of gellan and is predominantly in the potassium salt form. Acetan was isolated from fermentation broths of the cellulose minus mutant of *Acetobacter xylinum* strain CR1. Microbial growth conditions and the subsequent isolation and purification of acetan as the sodium salt are described elsewhere (22). The powdered samples were dispersed in water to a concentration of $1 mgmL^{-1}$, heated to 85-90°C for 1-2 hours whilst being stirred, and then cooled to room temperature. The xanthan preparations were centrifuged at 150,000g for 3 hours at room temperature in order to remove aggregates (microgels). The xanthan and acetan samples were then diluted to concentrations of $20\mu gmL^{-1}$ and $10\mu gmL^{-1}$ respectively. Gellan samples were diluted to various concentrations within the range $3\text{-}10\mu gmL^{-1}$.

Samples were prepared for the AFM by depositing a $2\mu L$ drop of the polysaccharide solution onto freshly cleaved mica surfaces and allowing it to dry in air for about 10 minutes. Uncoated samples were then imaged under 1-butanol (Sigma Chemicals). 1-Butanol is a suitable solvent for several reasons. Firstly, since 1-butanol is a precipitant for the polysaccharides it might reasonably be expected to inhibit desorption of the polysaccharides from the mica surface. Secondly, 1-butanol gives hysteresis free force-distance curves, with no marked adhesive element during retraction of the probe from the surface. This type of force-distance profile is essential if the applied force is to be controlled with sufficient precision during imaging. It has been shown that in order to obtain high quality images (20) it is necessary to operate the AFM in a narrow force window: if the imaging force is too low the contrast between molecule and substrate is lost, and if the imaging force is too high, the probe damages and displaces the polysaccharide (20). For metal coated samples the air dried deposits on mica were rotary shadowed with platinum/palladium alloy at a low angle (2°). These samples were also imaged under 1-butanol for comparison.

The AFM used in the present studies was an ECS (East Coast Scientific, Cambridge, UK) apparatus. The head, electronics and software were developed by Mark Welland, Martin Murrell and Tim Wong (Department of Engineering, Cambridge, UK) and the AFM is now marketed by ECS. Samples were contained in a liquid cell and imaged using constant force conditions. It has been noticed that the preset force may drift when scanning, particularly during the first 30 minutes after addition of liquid to the cell. The ECS instrument permits correction of the preset force during imaging. The short narrow variety of Nanoprobe cantilevers (Digital Instruments) were used with a nominal force constant of $0.38 Nm^{-1}$.

Results and Discussion

Figure 1 shows AFM images of metal coated xanthan (Figure 1a) and acetan (Figure 1b). The level of metal deposited is sufficient to pin down the molecules but insufficient to produce a continuous conducting layer on the mica surface. Images of the type shown in Figure 1 can be obtained extremely reproducibly. The samples can be stored and then re-imaged at a later time. The images show part of an entangled network of stiff elongated molecules. Individual molecules can be identified. The density of molecules within the network was found to increase with increasing polymer concentration although the coverage of the substrate is not completely homogeneous. The images of metal coated xanthan (Figure 1a) are consistent with reported STM (16, 17) and electron microscope (16) images of metal coated xanthan. Individual metal grains can be identified in Figure 1 and the grain size ($\approx$ 10nm) restricts the resolution of the image.

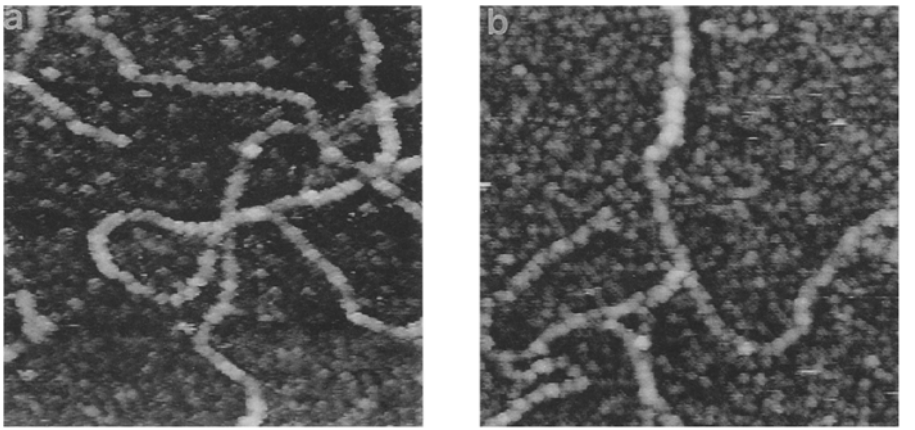

Fig. 1. AFM images of metal coated polysaccharides on mica. a. metal coated xanthan, scan size 700 x 700nm. b. metal coated acetan, scan size 400 x 400nm.

Figure 2 shows images of uncoated xanthan (Figure 2a) and uncoated acetan (Figure 2b). The images are of networks of stiff elongated molecules comparable with the images of metal coated samples. In the helical forms both xanthan and acetan are stiff molecules: xanthan has a Kuhn statistical segment length (l_k) $\approx$ 200nm (23) and acetan has $l_k >> 120$nm (24). The measured thickness of individual strands is larger than might be expected for the helical structures. The thickness varies depending upon the orientation of the strands with the scan direction; being largest perpendicular to the scan direction ($\sim$ 10nm) as compared to the expected helical thickness of $\approx$ 2nm (23). This is most likely due to the effect of probe broadening which arises because of the finite curvature of the apex of the probe tip. Similar effects are reported for studies on DNA, a helical biopolymer of similar dimensions, and the broadening effects can be reduced by the use of 'supertips' (25). Probe broadening effects can also be reduced if the molecules can be aligned and packed into ordered arrays (21). In this case the probe skips across the 'lattice' of molecules and only the apex of the tip contributes to the imaging process. This preliminary observation (21) is confirmed in Figure 3a which shows new data on aligned acetan chains. Within the arrays the measured strand thickness is $\approx$ 2nm which is typical for an individual helix. A periodic repeat of 5nm is observable along individual strands. X-ray fibre diffraction studies (6) have shown that acetan adopts a five-fold helical structure with a pitch of 4.8nm. Thus the periodic fluctuation along chains observed by AFM of uncoated molecules of acetan (Figure 3a) can be attributed to the helical structure of the polysaccharide.

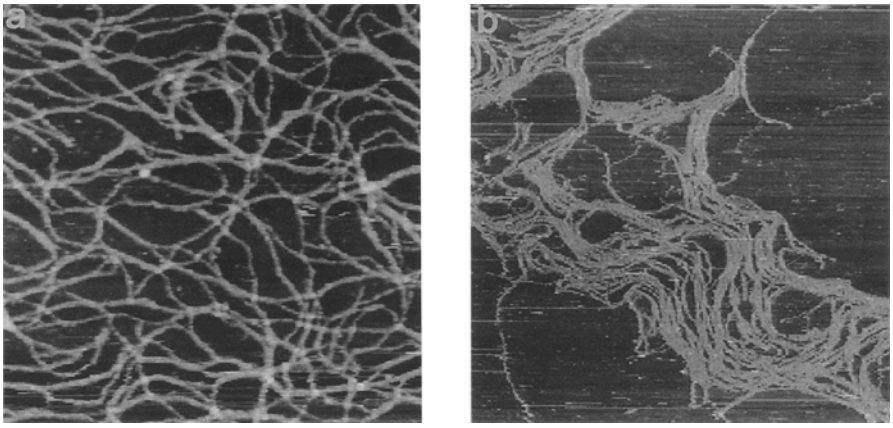

Fig 2. AFM images of uncoated polysaccharides on mica. a. xanthan, scan size 800 x 800nm. b. acetan, scan size 1200 x 1200nm.

Figure 3b shows AFM images of uncoated gellan samples. Whereas xanthan and acetan form entangled solutions upon concentration, gellan forms gels upon concentration and drying. Aligned fibres prepared from such aqueous preparations show crystalline x-ray diffraction patterns for gellan (7) whereas the patterns obtained for acetan and xanthan are non-crystalline (5, 6). The AFM image (Figure 3b) of gellan shows a continuous fibrous network within which it is not possible to identify individual polymers or polymer ends. The images of gellan differ from those of acetan and xanthan in several respects. The density of the observed gellan network appears to be constant irrespective of the original gellan concentration (in the range 3-10 μg mL^{-1}). Such an effect might be expected for a material which gels upon concentration to a critical concentration value. In addition larger forces were necessary to image gellan: the gellan samples were imaged at 10nN as compared to 3-4nN for acetan and xanthan. This may indicate formation of a more resilient structure for the gellan samples. The data shown in Figure 3b is believed to be the first AFM image of a polysaccharide gel network.

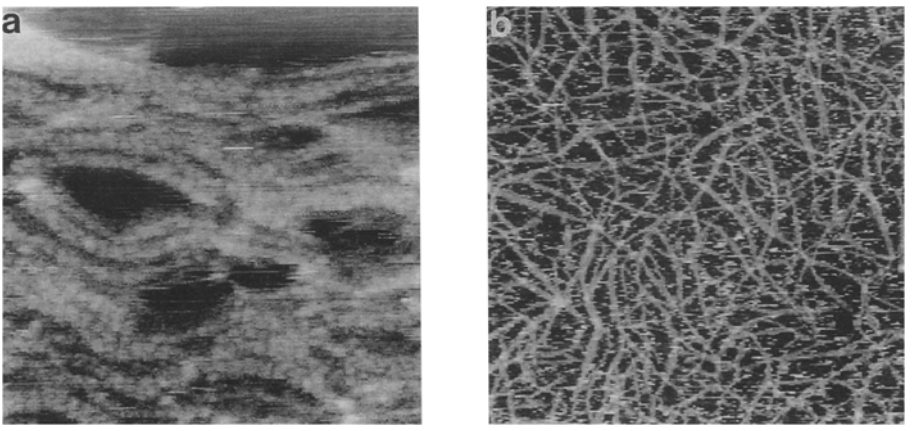

Fig. 3. AFM images of uncoated polysaccharides on mica. a. acetan, scan size 200 x 200nm. b. gellan gel, scan size 700 x 700nm.

This study demonstrates the superior resolution achievable through AFM studies of uncoated polysaccharides. It remains to be established whether the present methodology can be applied to study the architecture of more complex or branched polysaccharides.

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