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Visualisation of xanthan conformation by atomic force microscopy

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ABSTRACT

Direct visual evidence obtained by atomic force microscopy demonstrates that when xanthan is adsorbed from aqueous solution onto the heterogeneously charged substrate mica, its helical conformation is distorted. Following adsorption it requires annealing for several hours to restore its ordered helical state. Once the helix state reforms, the AFM images obtained showed clear resolution of the periodicity with a value of 4.7 nm consistent with the previously predicted models. In addition, the images also reveal evidence that the helix is formed by a double strand, a clarification of an ambiguity of the xanthan ultrastructure that has been outstanding for many years.

1. Introduction

Xanthan is a bacterial polysaccharide produced by *Xanthamonas campestris* (Garcia-Ochoa, Santos, Casas, & Gomez, 2000). The polysaccharide consists of a linear β(1,4) linked D glucose cellulosic backbone substituted with a regular trisaccharide sidechain, containing two mannose (Man) and a glucuronic acid (GlcA), attached on every other glucose at C-3. The charged sidechain consists of βDMan(1-4)βDGlcA(1-2)αDMan(1-. The terminal mannose units may contain a pyruvic acid substitute and the α-linked mannose units may have an acetyl group at position O-6 (Phillips & Williams, 2009). Another two recent studies have shown that there can be more heterogeneity of xanthan's repeat unit than was previously assumed in terms of the ratio of the charge groups within xanthan's sidechains depending upon the fermentation conditions: There are 6 different patterns of attachment of pyruvate and acetate groups to the pentasaccharide repeat unit, and the relative abundance of these affects the stability of the ordered structure (Kool, Gruppen, Sworn, & Schols, 2013; Kool, Gruppen, Sworn, & Schols, 2014). It is not clear whether this heterogeneity arises due to intra- or intermolecular substitution. The charged groups on the sidechains play a vital role in xanthan's aqueous solubility and also its structural conformation (Phillips & Williams, 2009). In the presence of stabilising counterions, which shield the intramolecular charge–charge interactions, the sidechains fold down compactly against the backbone leading to the formation of a 5-fold ordered helical structure (Norton, Goodall, Frangou, Morris, & Rees, 1984). The ordered structure is much stiffer than the disordered 'random coil' conformation. In the helical state xanthan has a persistence length in excess of 100 nm, ranking it amongst the stiffest known biopolymers. Previous studies have proven that electrostatic interactions between the charged groups within xanthan and screening counterions determine its ultrastructural conformation in solution (Matsuda, Biyajima, & Sato, 2009; Bejenariu, Popa, Picton, & Le Cerf, 2010; Brunchi, Morariu, & Bercea, 2014).

Traditional methods have been used widely to investigate the molecular conformation of polysaccharides. Optical rotation, circular dichroism, differential scanning calorimetry and rheology are convenient methods for following the course of disorder–order and order–disorder transitions in response to external variables (temperature, ionic strength, concentration of specific cations and denaturants). X-ray fibre diffraction remains the only technique capable of characterising ordered structures at atomic resolution, provided that the chains are well enough oriented and aligned, but atomic resolution has not yet been achieved for xanthan.

The principle question addressed by the present study is that there has been considerable ambiguity for many years on the detail of xanthan's secondary structure (Morris, 1998). The initial interpretation of X-ray fibre diffraction data was that it formed a single helix (Moorhouse, Walkinshaw, & Arnott, 1977). A subsequent study (Okuyama et al., 1980), carried out in response to the "two strands = double helix" lobby, examined possible double-helix

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models. It was concluded that, on the basis of the X-ray evidence alone, it was not possible to assign a double-helix or single helical model for xanthan.

In terms of physical chemical studies the salt-induced disorder–order transition followed first order rather than second order kinetics, which suggested a single helix (Norton et al., 1984). Many groups (Morris, 1998) have equated observed dimerization of xanthan with double-helix formation; but that is not evidence based and it is potentially an oversimplified interpretation. The ambiguity with such methods is the fact that they are *ensemble* measurements. This means that the analytical conclusion is controlled by the ratio of ordered to disordered states, so that they lack a certain degree of sensitivity compared to microscopical techniques, such as atomic force microscopy (AFM). AFM is capable of visualising the structure of individual molecules. The main objective of this study is to provide direct evidence on the nature of xanthan's secondary structure. The unique advantage of AFM is its ability to visualise directly the topology of polymer networks under near-native conditions, which can be a very powerful complimentary technique to combine with *ensemble* methodologies. An integral study using various biophysical techniques, namely, AFM, gel permeation chromatography with multi-angle light scattering (GPC-MALLS) and intrinsic viscosity measurements by capillary viscometry on the conformation of xanthan, following various different treatments (heating, autoclaving, irradiation and high pressure homogenisation), was recently reported (Gulrez, Al-Assaf, Fang, Phillips, & Gunning, 2012). Several polymer parameters derived from these techniques, such as the radius of gyration (Rg), M_W, polydispersity, molar mass per unit contour length of the rod (M_L) and Huggins constant (K_H) were correlated well with the results obtained by AFM. It was possible to correlate the height measurements obtained by AFM with values close to 1000 Dalton per nanometre ($Da\,nm^{-1}$) and 2000 ($Da\,nm^{-1}$) assigned for single and double helix, respectively in agreement with previous reports which solely relied on light scattering measurements (Sato, Kojima, Norisuye, & Fujita, 1984; Sato, Norisuye, & Fujita, 1984). Furthermore, using positively-charged mica (coated with poly-L-lysine) a single strand molecule was trapped in a 'random coil' conformation (Gulrez et al., 2012). This is consistent with the widely agreed view that xanthan at low concentration and negligible ionic strength adopts 'random coil' conformation.

The present study reveals new images of xanthan at sub-molecular resolution revealing the fine detail of its secondary structure development, which has enabled the process of charge screening to be investigated in a manner never previously reported.

2. Experimental

2.1. Atomic force microscopy

The atomic force microscope (Cypher AFM, Asylum Research Inc, an Oxford Instruments company, Santa Barbara, CA, USA) was operated in AC mode in aqueous buffers containing different counterions. Buffer 1: 10 mM HEPES 3 mM $ZnCl_2$ pH 5.3, and buffer 2: 10 mM HEPES 3 mM $NiCl_2$ pH 7.0 (Sigma-Aldrich Chemical, Poole, Dorset, UK). Oscillation of the cantilevers at their fundamental resonant frequency was driven using 'blueDrive' photo-thermal excitation. Photothermal excitation is a new technology developed by Asylum research that provides a more stable and controlled form of cantilever oscillation. This is achieved by positioning a laser beam with wavelength 425 nm and modulated power directly onto the cantilever's bimetallic strip, as opposed to the traditional piezo-acoustic method, which mechanically oscillates the tip holder causing more potential disturbance of the samples. Localised heating by the blue laser causes the cantilever to bend due to the bimetallic strip effect and modulation of the power causes the probe to oscillate at an accurately controlled frequency and amplitude. The laser power modulation frequency was set at the fundamental resonant frequency of the cantilever (1.37 MHz) and the power level 124.8 μW set to generate an appropriately small oscillation amplitude (~1 nm). The feedback loop control set-point was also kept at a very low level of damping of the cantilever's free oscillation (~5–10%) to minimise the loading force on the molecules. The AFM tips used were Arrow UHF-AuD (NanoWorld AG, Neuchâtel, Switzerland). Scan rates were set at 1.5 Hz.

2.2. Preparation of xanthan solutions

The xanthan used in this study was a powdered food grade xanthan gum (Keltrol RD, CP Kelco, Atlanta, GA, USA). 'RD' stands for a readily dissolvable product. The stock solution was prepared at a concentration of 1 $mg\,ml^{-1}$ in pure water (18.2 MΩ). The xanthan powder was dispersed immediately after addition to the water at 22 °C by stirring. It was then left for 30 min to hydrate before heating to 95 °C for 60 min to completely disperse the molecules. The stock solution was allowed to cool to room temperature (22 °C) and then diluted to 3 $\mu g\,ml^{-1}$ into either water (method 1, below), or the aqueous buffers (method 2, below). The diluted solutions were then re-heated to 95 °C for 60 min to reduce any aggregation and allowed to cool back to 22 °C prior to the AFM imaging preparation procedures.

The additional heating step was merely to ensure that the same structure and proportions of soluble/aggregate fractions are present in the test material (renatured state). Gulrez et al. (2012) investigated the effect of heat treatment on xanthan aqueous solutions (4 mg/mL) dissolved in distilled water, which was subsequently diluted to contain 0.1 M LiNO3 prior to injection into the GPC-MALLS system. They demonstrated that heating xanthan aqueous solution up to 40 min at 85 °C resulted in similar molecular weight parameters (i.e. weight average molecular weight, % mass recovery and polydispersity). Further heating up to 60 min resulted in an increase in the mass recovery and a slight increase in the molecular weight as a result of disassociation of large aggregates initially retained on the 0.45 μm filter. The molecular weight is reduced to almost half with full mass recovery and an increase in the polydispersity (from 1.64 to 2.75) when the diluted solution was heated for 2 at 85 °C.

2.1. AFM imaging preparation procedures

Two methods were used to physisorb xanthan molecules onto freshly-cleaved muscovite mica (Agar Scientific, Cambridge, UK).

2.2.1. Method 1: drop deposition (includes drying)

A 3.5 μl droplet of xanthan at a concentration of 3 $\mu g\,ml^{-1}$ in water was placed onto the freshly-cleaved mica and left to evaporate at room temperature (22 °C). When fully dry the sample was then placed into the liquid cell of the AFM and imaged in the aqueous buffers described in Section 2.1.

2.2.2. Method 2: in-situ adsorption (no-drying)

100 μl of the buffer-diluted xanthan solution (3 $\mu g\,ml^{-1}$) was placed directly into the liquid cell of the AFM, which contained freshly-cleaved mica and then imaged as described in Section 2.1.

3. Results

Fig. 1 displays an example of the data that were always obtained at the early stages of imaging xanthan in aqueous buffers, prepared by both methods (Fig. 1a drop deposition, Fig. 1b *in-situ* adsorption). The swirly white lines over one molecule in each image