Note

Single Molecules and Networks of Xanthan Gum Probed by Atomic Force Microscopy

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Nano-scale structures of individual single molecules and supra-molecular networks of xanthan gum were investigated using atomic force microscopy (AFM). AFM images of xanthan dissolved in water at ambient temperatures revealed branched fibrous structures, the cross-sectional dimensions of which typically ranged from 1 to 1.5 nm. Individual molecular chains, the cross-sectional dimensions of which were around 0.5 nm, were observed after heating at 90°C. Renaturation behavior upon successive cooling was dependent on the concentration of xanthan and the temperature. At 60°C, the formation of an anti-parallel double-stranded helix from an isolated molecule was evident. Air-drying of aqueous solutions containing either 1 or 10 μg/mL xanthan at room temperature resulted in the formation of nanometer- or micrometer-sized networks, respectively. These networks appeared to be composed of multiple filaments aggregated in parallel alignment to form thicker bundles with varied thicknesses, typically ranging from 0.5 to 2 nm.

Keywords: Anti-parallel double-stranded helix, atomic force microscopy, denaturation, network, renaturation, single molecule imaging, xanthan gum

Introduction

Xanthan gum is the extracellular polysaccharide secreted by the Xanthomonas campestris bacterium in the form of a hydrated slimy layer covering the cell (Morris, 1998). Xanthan has a cellulosic backbone consisting of 1,4-linked β-D-glucose, but is soluble in water under ambient conditions due to the presence of bulky trisaccharide side groups attached to alternate glucose residues of the backbone (Jansson et al., 1975). Aqueous solutions of xanthan exhibit extremely high viscosities at low shear rates, rapid reductions in viscosity with increasing shear rate and gel-like responses to small amplitude oscillatory deformations (Choppe et al., 2010). These rheological properties make xanthan a desirable stabilizer or rheology modifier in various applications. Xanthan is thus commercially manufactured and utilized in the food, oil drilling, personal care and pharmaceutical industries.

The commercial production of xanthan often involves a heat treatment to inactivate microorganisms (Smith and Pace, 1982). Xanthan molecules are believed to be in the double-stranded helical conformation in the native state, while structural order is lost at temperatures above its conformational transition temperature (Tc) (Morris, 1998). Heat-denatured molecules renature upon cooling to a temperature below Tc; however, an aqueous dispersion of renatured xanthan may exhibit significant increases in viscosity and shear modulus relative to the dispersion prior to heating (Ovitt and Brant, 1994; Capron et al., 1998a). Sato and co-workers recently studied thermal denaturation and renaturation behavior of xanthan using a light scattering technique and revealed that at relatively high xanthan concentrations, the apparent molecular weight in the renatured state can be much larger than that in the native state due to aggregation between multiple molecules occurring during the renaturation process (Matsuda et al., 2010).

Atomic force microscopy (AFM) has been shown to be a useful tool for investigating individual molecules and aggregated networks of biopolymers (Ikeda et al., 2001; 2004; 2005; Ikeda and Morris, 2002; Abu-Lail and Camesano, 2003; Ikeda and Shishido, 2005; Morris et al., 2009). The
first reported AFM images of xanthan were obtained by imaging under 1-butanol using the constant force or direct current (DC) mode (Kirby et al., 1995), while the feasibility of alternating current (AC) mode imaging in air was soon verified (Gunning et al., 1996). Comparisons between AFM images of unheated and renatured xanthan molecules in dilute conditions suggested the occurrence of irreversible disaggregation during heating (Capron et al., 1998b). Statistical analysis of AFM images of xanthan molecules renatured under dilute conditions indicated a systematic reduction in the flexibility of the molecular chain with increasing salt concentration (Camesano and Wilkinson, 2001).

The objective of the present study was to gain more insight into thermally induced denaturation and renaturation behavior of xanthan. Xanthan was rehydrated in an aqueous salt solution, heated or unheated, air-dried on a solid substrate, and imaged in air using AFM operating in AC mode. Not only dilute conditions but also more concentrated conditions were investigated due to their relevance to practical applications.

### Materials and Methods

Xanthan gum was provided by CP Kelco (San Diego, CA) in the form of dried powders. Xanthan powders were dispersed at a concentration of 1 mg/mL in a 0.01 mol/dm$^3$ aqueous solution of NaCl and moderately stirred using a magnetic stirrer overnight under ambient conditions. A portion of the xanthan solution was directly diluted to give a xanthan concentration of 1 μg/mL using 0.01 mol/dm$^3$ NaCl solution. Another portion of the xanthan solution was heated at 90°C for 30 min and cooled to room temperature before being diluted to a xanthan concentration of 1–10 μg/mL using the 0.01 mol/dm$^3$ NaCl solution. A 2-μL volume of the diluted solutions was drop-deposited onto a freshly cleaved mica surface and allowed to stand in a covered Petri dish for at least 15 min until no liquid was visible on the mica surface.

For imaging individual single molecules, the heated xanthan solution was diluted to a xanthan concentration of 1 μg/mL using pre-heated distilled water and cooled to approximately 70°C. A 2-μL volume of the diluted solution was drop-deposited onto a freshly cleaved and pre-heated mica surface and allowed to stand at 60°C until no liquid was visible on the mica surface.

AFM imaging was performed under ambient conditions using a multimode imaging unit SPA-400 (SII NanoTechnology Inc., Chiba, Japan) operated in dynamic force (AC) mode using a probe station SPI3800N (SII NanoTechnology Inc.). Samples were scanned in air at a scan rate of 0.5–1 Hz using a beam-shaped silicon cantilever, the nominal spring constant and resonance frequency of which were given as 12 N/m and 136 kHz, respectively. Topographical data were stored in a 256 × 256 or 512 × 512 pixel format, and were processed using NanoNavi II software (SII NanoTechnology Inc.) to flatten the background. Sample preparation was performed in triplicate and 10–20 images were obtained for each preparation. Therefore, the reported images represent approximately 50 images obtained for each condition.

### Results and Discussion

The sample preparation procedure adopted in this study is reasonably mild, but influences the images obtained. Three-dimensional polymer assemblies in a sample solution will be flattened into two-dimensional objects on the substrate. Air-drying will lead to an increase in the polysaccharide concentration and hence promote the assembly of polysaccharide molecules. The aim of the present study is to examine the structure of such polysaccharide assemblies.

Xanthan is commonly recovered from a fermented broth in the form of fibrous precipitate by the addition of water-miscible lower alcohol such as ethanol and isopropyl alcohol (Smith and Pace, 1982; Albiter et al., 1994). Accordingly, recovered xanthan molecules are no longer in the native state but aggregated due to alcohol-induced desolvation (Morris et al., 2001). Figure 1a shows a topographical AFM image of xanthan rehydrated to give a concentration of 1 μg/mL in

![Fig. 1.](image)
0.01 mol/dm$^3$ NaCl at room temperature and air-dried on the mica surface at room temperature. These molecules should not have undergone conformational transitions during preparation, as $T_c$ of xanthan in 0.01 mol/dm$^3$ NaCl is much higher than room temperature (Callet et al., 1987). The conformational transition of xanthan is known to exhibit a low degree of cooperativity and high sensitivity to the salt concentration (Rinaudo, 2004). Based on the reported data on the optical rotation (Callet et al., 1987) and dynamic moduli (Choppe et al., 2010), the conformational transition of xanthan in the presence of 0.01 mol/dm$^3$ NaCl is estimated to occur in a broad temperature range approximately from 50 to 80°C and from 60 to 80°C, respectively. The unheated xanthan revealed seemingly stiff fibrous structure with branches (Fig. 1a). The cross sectional heights of the fibrous structure varied along the axis, typically ranging from 1 nm to 1.5 nm (Fig. 1b). These values are 2 – 3 times larger than the reported value (0.48 nm) of the cross sectional height of a single molecular chain of xanthan evaluated using AFM (Camesano and Wilkinson, 2001). These results suggest that multiple molecular chains remain to be aggregated in parallel alignment in unheated aqueous solutions of xanthan. Variations in the cross sectional height in the direction of the fiber axis indicate variations in the number of aggregated molecular chains in a cross section and/or uniaxial anisotropy (non-circular cross section) of the fibrous structures.

In order to ensure complete denaturation, xanthan solutions were heated at 90°C for 30 min and then diluted using pre-heated (ca. 70°C) distilled water to have a xanthan concentration of 1 μg/mL and a negligible NaCl concentration (10$^{-3}$ mol/dm$^3$). The heat-denatured xanthan solutions were then deposited onto the mica surface maintained at 60°C because drying at higher temperatures yielded irreproducible images. Figure 2a confirms that single xanthan molecules can be visualized based on the present sample preparation procedure. The overall dimensions of individual molecules are much smaller than that of unheated xanthan shown in Fig. 1a. The cross sectional heights were fairly constant around 0.5 nm, consistent with the literature value for a single molecular chain of xanthan (Camesano and Wilkinson, 2001).

These molecules are considered to be partially renatured, as renaturation of xanthan upon cooling is expected to start around 70 – 80°C and finish around 50°C in the absence of added salt (Choppe et al., 2010). Matsuda et al. (2009) reported that the molar mass distribution of xanthan renatured under dilute conditions showed good agreement with that of fully dissociated unimers, but that the molar mass dependence of the radius of gyration agreed with that of double-stranded helices. These results suggest that individual renatured molecules form anti-parallel double-stranded helices with a single-stranded hairpin loop at one end in dilute conditions (Capron et al., 1997). The presence of an intra-molecularly associated anti-parallel double-stranded helix with a single-stranded loop is evident in Fig. 2a. Figures 2b and 2c show that the cross sectional heights of the double-stranded helix and the single-stranded loop are 0.87 nm and 0.50 nm, respectively. These values agree well with the reported cross sectional height of a double-stranded helix (0.86 nm) and a single-stranded helix (0.48 nm) of xanthan determined using AFM (Camesano and Wilkinson, 2001).

In order to examine the fully renatured structure, heat-denatured xanthan solutions were diluted in 0.01 mol/dm$^3$ NaCl and air-dried on the mica surface at room temperature. Figure 3a shows that localized networks spanning nanometer-scale areas were formed after air-drying of a 1 μg/mL solution of fully renatured xanthan. It also appears that long (> 1 μm) and tenuous branches extend from these networks and link neighboring networks. These networks are likely to develop during air-drying due to an increased xanthan concentration, and eventually collapse and flatten on the mica surface. The air-dried mica surface is covered with a thin (~ 1 nm) layer of water under ambient conditions (Beagleshole and Christenson, 1992). Based on the droplet volume before drying (2 μL), wet area on mica (~ 1 cm$^2$), and the thickness of water on mica after drying (~ 1 nm), the effective concentration of xanthan shown in Fig. 3a is estimated to be in the order of 1% w/w. It is thus plausible to assume that renatured xanthan
molecules in a relatively concentrated solution are not homogeneously dispersed but form localized networks linking one another with tenuous branches. It can be also hypothesized that shear-thinning properties of xanthan sols (Choppe et al., 2010) are attributed to disconnection, deformation and dissociation of localized networks with increasing shear rate and restoration of the initial network structure with decreasing shear rate.

The difference between the AFM image prior to heating (Fig. 1a) and that after heat-denaturation and renaturation (Fig. 3a) confirms that heating causes irreversible structural changes. Rheological data recorded during repeated heating-cooling treatments of an aqueous dispersion of xanthan frequently reveal thermal hysteresis only in the first heating-cooling cycle (Oviatt and Brant, 1994; Capron et al., 1998a). It is therefore considered that the initial unheated state differs from the renatured state and that transitions between the denatured and renatured states are thermo-reversible.

Air-drying from a 10 μg/mL solution of fully renatured xanthan was found to induce the formation of micrometer-sized networks on the mica surface (Fig. 3b). As no ends of fibrous structures are visible in this image, it is difficult to distinguish individual molecular chains. It also appears that the strand thickness is uneven. A magnified view (Fig. 3c) reveals that multiple filaments are aligned in parallel and aggregated to form network strands with varied thicknesses. The height profile (Fig. 3d) shows that the strand thickness varies typically from 0.5 nm to 2 nm. Matsuda et al. (2009) suggested from light scattering studies that double-stranded helices of xanthan unwind from both ends upon heating, but remain as dimers under non-dilute conditions (> 1% w/v) due to incomplete unwinding. Upon renaturation, a network develops as double-stranded helices are formed by pairing two unwound chains belonging to two different dimers. This type of network is expected to show a uniform strand thickness that corresponds to the diameter of the cross section of individual double-stranded helices. The present study suggests an additional mode of network formation of xanthan that does not require incomplete unwinding into dimers upon denaturation, but involves helix-helix aggregation upon renaturation.

Conclusions

AFM has been shown to have unique capabilities to probe the nano-scale structure of single molecules and supra-molecular assemblies of xanthan. Unheated xanthan molecules were confirmed to be in aggregated states. These aggregated structures were not reconstituted upon renaturation after they were destroyed by heating over the conformational transition temperature. Single molecular imaging of partially renatured
molecules provided direct evidence in support of the formation of double-stranded helices. Networks having various strand thicknesses developed from fully renatured molecules through aggregation of various numbers of double-stranded helices aligned in parallel. Structural information obtained in the present study will serve as a foundation for understanding the rheological characteristics of xanthan.

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References


