Morphological Imaging of Single Methylcellulose Chains and Their Thermoresponsive Assembly on a Highly Oriented Pyrolytic Graphite Surface

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Individual methylcellulose (MC) chains and their thermoresponsive assembly were successfully visualized on a highly oriented pyrolytic graphite (HOPG) surface by atomic force microscopy (AFM). Momentary contact of a dilute MC solution at 4 °C onto the HOPG substrate permitted clear imaging of individual MC chains having a molecular thickness of ca. 0.5 nm and a hexagonal orientation. By increasing the solution temperature from 4 to 80 °C, it was possible to bring about significant changes in the MC nanomorphology from stretched molecular chains to disordered massive aggregates. It was presumed that the hydrophobic interaction between the MC chains and the HOPG π-conjugated system led to the successful visualization of thermally responsive changes in the MC conformations. These results imply that HOPG substrates could be used for clear nanoimaging of cellulosic polymers and other structural polysaccharides.

Introduction

Methylcellulose (MC) is one of the typical functional biomacromolecules derived from cellulose. Water-soluble MC having a moderately low degree of substitution (DS) with methyl groups is extensively used as a binder and thickener in food, cosmetic, and pharmaceutical applications.[1–6] The MC polymer is industrially produced through the direct etherification of solid-state cellulose with a methylation reagent, resulting in a heterogeneous molecular structure consisting of amphipathic anhydrous glucopyranose (AHG; hydrophilic equatorial side and hydrophobic axial plane) and hydrophobic methylated units.[7] Recently, the MC polymer has attracted much attention due to its thermoresponsive, reversible sol–gel transition around a specific lower critical solution temperature (LCST).[1,2] As such, many researchers have studied the thermodynamics of MC polymers in order to prepare smart materials for applications in bio and nanoengineering fields.[8–10] To date, this unique gelation mechanism has been investigated by viscoelastic and thermoanalytical approaches, and it has been proposed that the gelation of MC polymers in water is involved in the hydrophobic association and microphase separation of the heterogeneously methylated units in the MC chains by heating.[1–6] Further understanding of the MC molecular dynamics is required for the advancement of functional polymer design; however, the thermally responsive morphology of MC polymers at the single molecular level has not yet been investigated in detail. The first AFM experiments on the dynamics of single polymer chains were performed by Minko et al.; drastic conformational changes of polyelectrolytes were successfully visualized on mica substrates at different pH and salt conditions.[11] Since then, the nanodynamics of various synthetic polymers have been elucidated via molecular imaging.[12–14]

The molecular imaging of single polymer chains gives valuable information with respect to the fundamental perspectives for polymer design.[15,16] Atomic force microscopy (AFM) has attracted great interest as a powerful tool for real visualization at the atomic/monomolecular level, especially in the *Z*-direction, giving conformational images and information on nanodynamics of individual polymer chains on atomically flat substrates.[15–17] Hence, AFM is beneficial for resolving the individual shapes of macromolecules and their assembly, whereas conventional analytical methods provide only averaged information on polymer populations, for example, light scattering, viscoelastic, thermodynamic, and spectroscopic characteristics.[18–20]

Many researchers have reported that appropriate interactions at the polymer/substrate interface play a key role in obtaining highly resolved AFM images without any sample displacement, distortion, or artifacts.[15,16] Cleaved mica and highly oriented pyrolytic graphite (HOPG) are two of the more commonly used substrates in the AFM imaging of hydrophilic and hydrophobic polymers, respectively, due primarily to their atomic smoothness. For instance, DNA chains and various water-soluble biopolymers were successfully visualized on a negatively charged mica surface through electrostatic screening via cationic mediators.[21–23] It has also been reported that *n*-alkane-grafted polymers adopt a regular hexagonal orientation on an HOPG crystal surface via hydrophobic interactions.[24,25]

AFM imaging of water-soluble polysaccharides has been widely studied using mica as a substrate.[26–28] However, a number of poorly resolved AFM images have been produced by conventional methods. Recently, Hatakeyama et al. observed the thermoresponsive conformational changes in MC polymers cross-linked with urethane on mica by using an AFM technique,[29] however, few monomolecular-level MC chains were observed. MC-type polymers have an amphipathic structure composed of both AHG and methylated units in the polymer backbone. Thus, even though an MC polymer with an appropriate DS is highly soluble in water, the hydrophilic mica surface is expected to induce MC aggregation due to the weak affinity

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of this polymer for the substrate surface. We have recently reported highly resolved AFM images of carboxymethylcellulose (CMC), a water-soluble cellulose derivative, by using a hydrophobic HOPG substrate.[30] CMC is a heterogeneously CM-substituted cellulosic polymer similar to the MC-type polymers with a heteroamphipathic molecular structure. The conformational changes in individual CMC chains were successfully visualized on the HOPG surface by using a dilute CMC solution under various salt conditions. In our recent study, the first-time molecular imaging of individual cellulose chains was also achieved by using the HOPG substrate and a cellulose/Cu-amine solution.[31] Cellulose consists only of AHG units and has strong molecular assembly features; but the adequate interaction with the HOPG surface enabled the highly resolved visualization of individual cellulose chains. These results suggested that some CH-π interaction between the cellulosic molecules and the HOPG π-conjugated system may have contributed to the moderate molecular chain attachment onto the HOPG surface for distinct molecular imaging.

In this study, the conformational changes in individual MC chains were visualized on an HOPG surface by tapping-mode AFM analysis under atmospheric conditions. The molecular dynamics of single MC chains and their assembly were investigated by changing the temperature of the dilute aqueous MC solution placed in contact with the HOPG surface. The morphological images obtained are also discussed with regard to the rheological properties of the MC solution.

Materials and Methods

Materials. MC powders (DS: ca. 1.8; molecular weight, MW: ca. 2×10^4 and ca. 6×10^4 g mol^{-1}) were purchased from Wako Pure Chemical Industries, Co. Ltd., Japan; the latter was called longer-chain MC. Atomically flat HOPG plates (Veeco Instruments, Inc.) were used as substrates for AFM observation. The water used in this study was purified with a Milli-Q system (Millipore, Inc.). Other chemicals were extra-pure reagent grade and used without further purification.

Sample Preparation. A dilute MC solution (10 mg L^{-1}) prepared using Milli-Q water was first heated to over 80 °C, and then stored at 4 °C for more than 48 h. Ten μL of MC solution adjusted to 4°, 25°, 55°, or 80 °C was added dropwise onto a cleaved and acetone-prewashed HOPG substrate at room temperature and immediately removed within 3 s by blowing off, followed by thorough rinsing with Milli-Q water and drying in a stream of dry nitrogen gas. In the case of longer-chain MC, the polymer concentration and the contact time were adjusted to 5 mg L^{-1} and 5 s, respectively.

AFM Imaging. AFM observation was performed under ambient conditions using a NanoScope IIIa atomic force microscope (Veeco Instruments, Inc.) operated in tapping mode with an E-type piezoelectric scanner and Tap300 metrology probes (single-crystal silicon cantilevers; length: 125 μm, curvature radius: 5–10 nm, spring constant: 40 N m^{-1}, resonance frequency: 200–400 kHz, Veeco Instruments, Inc.). AFM measurements were carried out in five different regions (scan size: 1.0×1.0 and 3.0×3.0 μm^2) per sample. The morphological data of the AFM images were analyzed using the AFM-accessory software (version 5.12b36). The thickness of MC chains was averaged along the individual chain fragments on the atomically flat areas ($n = 50$). All AFM images presented here were third-order flattened through the removal of the vertical offset between scan lines using the software to correct the distortion at the micrometer scale, but no other digital operation was performed.[30,31]

Rheological Analysis. A cone-plate-type rheometer, Rheosol-G2000 (UBM Co. Ltd., Japan), was used for determining the dynamic viscoelasticity of the aqueous MC solution. Both radii of the cone with an angle of 2° and the plate were 50 mm. The rheometer was equipped with a reservoir to prevent drying of the sample during the measurements. The viscoelastic data were recorded at solution temperatures ranging from 25 to 80 °C. Isothermal frequency sweeps (ω: 0.1–100 rad s^{-1}) were performed at each temperature with a strain amplitude of 5%.

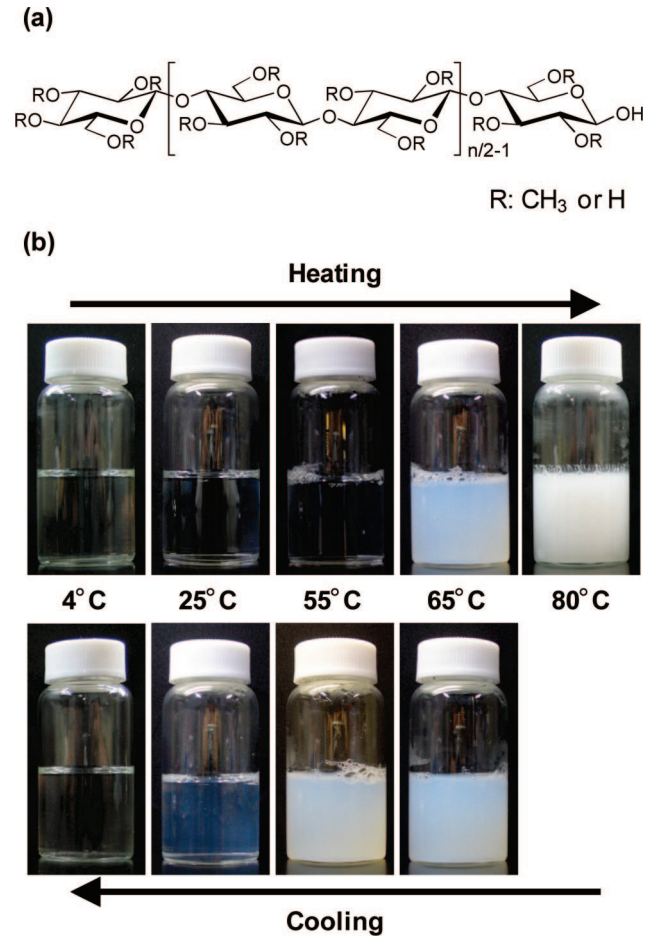

Figure 1. Chemical structure of an MC molecule composed of original AHG units (R: H) and methylated glucopyranose units (R: CH_3 or H) (a) and optical images of an aqueous MC solution (MW: 2×10^4 g mol^{-1}, concentration: 10 g L^{-1}) at different solution temperatures in both heating and cooling processes (b).

Results and Discussion

AFM Imaging of Single MC Chains. The molecular structure of MC and the reversible gelation behavior of an aqueous MC solution (10 g L^{-1}) during both heating and cooling processes are illustrated in Figure 1. The DS values of the MC used in this study were ca. 1.8, averaging less than two methyl groups per AHG unit. However, the hydroxyl groups of original cellulose are heterogeneously etherified through the solid-phase MC preparation, and thus the MC molecules have both original AHG and various methylated units located in the main polymer chain.[7] The MC solution rapidly became cloudy at ca. 65 °C in the heating process, and the white cloudy gel gradually returned to the original transparent solution state during the cooling process as shown in Figure 1b. This phenomenon corresponds to the typical reversible gelation behavior of the MC solution. Thus, the LCST of 10 g L^{-1} MC aqueous solution was estimated to be ca. 65 °C by conventional visual inspection.

In general, water-soluble polymer chains can be easily placed on a hydrophilic mica surface by simply applying the polymer solution dropwise, and in most cases, AFM observation is achieved by controlling the solution conditions.[19] However, the

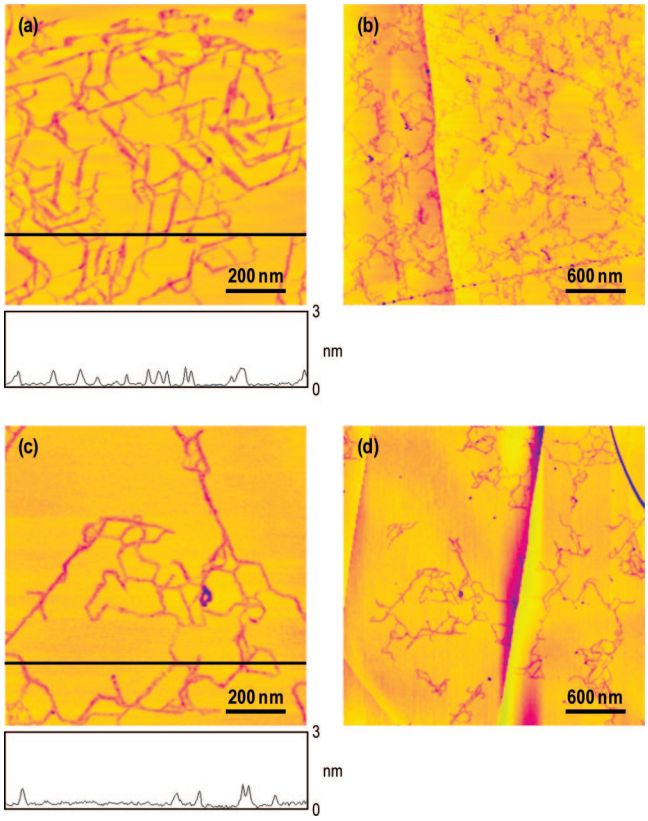

Figure 2. AFM images of MC chains on an HOPG substrate treated with an aqueous MC solution at 4 °C. MW: 2×10^4 g mol^{-1} (a,b) and 6×10^4 g mol^{-1} (c,d). Image size: 1.0×1.0 μm^2 (a,c) and 3.0×3.0 μm^2 (b,d). Height profiles along the scanning (black) lines are indicated below each AFM image.

conventional method was not effective for the imaging of single molecules of various polysaccharides such as hyaluronan,[32] CMC,[30,33] and modified MC[29] despite these polymers being homogeneously soluble in water. In the case of unmodified MC, highly resolved AFM imaging was not possible using mica (data not shown). Hence, the hydrophobic HOPG substrate was used for immobilization of the MC chains for AFM imaging according to our previous CMC and cellulose studies.[30,31]

Parts a and b of Figure 2 show AFM topographical images of HOPG substrates treated with a 10 mg L^{-1} aqueous MC (MW: ca. 2×10^4 g mol^{-1}) solution at a homogeneous dissolution temperature of 4 °C. Single MC chains were successfully observed on the HOPG surface, suggesting that the water-soluble but partially hydrophobic MC molecules were attached well to the hydrophobic HOPG surface, under the same conditions as those used in the unsuccessful attempts to attach identical MC molecules to a mica surface. In our previous CMC study,[30] the amount of contact time between the sample droplet and the HOPG substrate surface was crucial for the clear imaging of individual CMC chains; a shorter contact time of less than 3 s resulted in a better molecular resolution. In all cases for the MC imaging, contact times that were too long produced an excess of MC polymers on the substrate surface, resulting in obscure imaging of the accumulated MC chains (data not shown). The same contact time of less than 3 s was adopted in the present study. The MC chains had an average height of 0.51 ± 0.10 nm ($n = 50$) and were slightly thicker but roughly fitting to the height in the axial direction of the cellulosic AHG unit, as estimated from the space filling model and crystal geometry.[34] The difference in thickness might be attributed to the heterogeneous backbone structure of the MC polymer

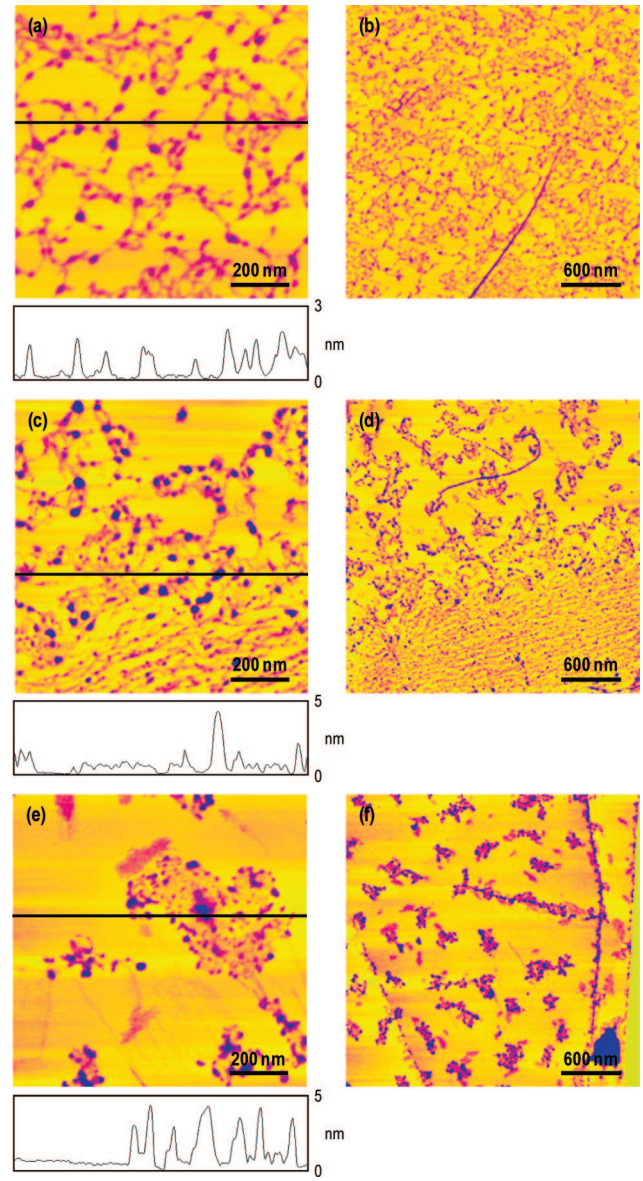

Figure 3. AFM images of MC chains on an HOPG substrate treated with an aqueous MC solution. MC solution temperatures: 25 °C (a,b), 55 °C (c,d), and 80 °C (e,f). Image size: 1.0×1.0 μm^2 (a,c,e) and 3.0×3.0 μm^2 (b,d,f). Height profiles along the scanning (black) lines are indicated below each AFM image.

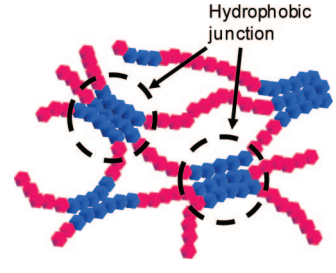

Figure 4. Schematic illustration of the thermoresponsive microphase separation of MC molecules through the hydrophobic association of the highly methylated units at temperatures greater than the LCST: hydrophobic (blue) and hydrophilic (red) units.

consisting of AHG and methylated units. Single MC molecules deposited onto the HOPG surface demonstrated a unique ordered morphology in which molecules appeared to overlap each other crosswise at ca. 120° (Figure 2a,b). This suggests that the MC chains may be aligned on the hexagonal HOPG crystal surface

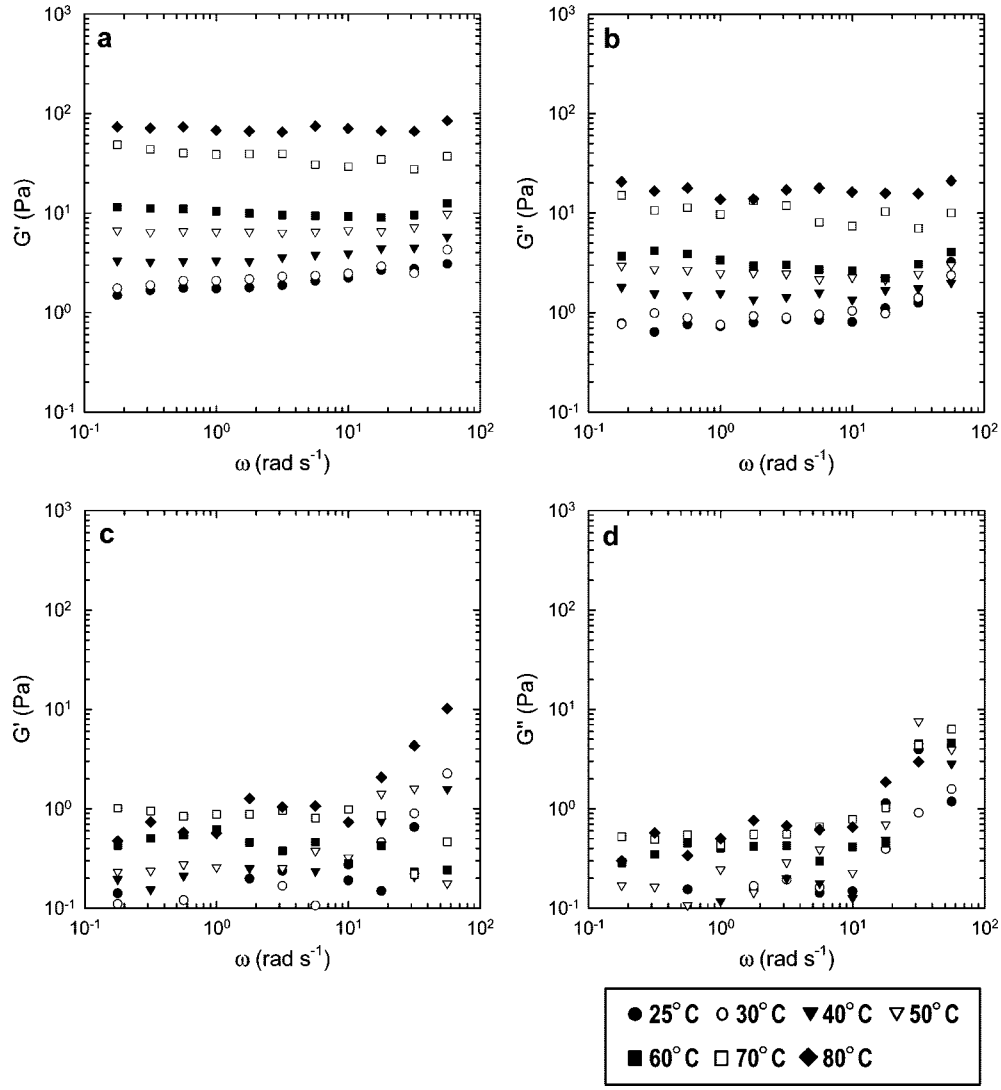

Figure 5. Dynamic share storage modulus *G′* and loss modulus *G″* curves of aqueous MC solutions at solution temperatures ranging from 25 to 80 °C, at frequency $\omega = 0.1$–100 rad s^{-1}. MC concentration: 10 g L^{-1} (a,b) and 10 mg L^{-1} (c,d).

through hydrophobic interactions between the MC molecules and the π-conjugated system of the HOPG crystal plane.

The AFM images of the HOPG surface treated with a dilute longer-chain MC solution (MW: ca. 6×10^4 g mol^{-1}) at 4 °C also display the well-ordered morphology consistent with hexagonal alignment, as shown in Figure 2c,d. In this case, the MC concentration and the contact time were set at 5 mg L^{-1} and 5 s, respectively, for the clear visualization of individual MC chains. The average thickness of longer-chain MC corresponded approximately to the single molecular size of MC chains (0.52 ± 0.12 nm, $n = 50$). Thus, the individual MC molecules were successfully visualized on the HOPG surface by using a cooled MC solution in which the concentration of polymer solution and the contact time between the MC droplet and the HOPG substrate were regulated.

Morphological Changes in MC Chains Induced by Heating. It is well-known that a white cloudy MC gel forms at temperatures higher than its LCST, and in this study, the MC (MW: ca. 2×10^4 g mol^{-1}) solution of 10 g L^{-1} changed from transparent sol to cloudy gel phases via heating above 65 °C and it gradually returned to a clear sol state via cooling, as shown in Figure 1b. Herein, the AFM investigation was carried out with regard to the molecular conformation of the MC chains deposited onto the HOPG surfaces at different solution temperature conditions (25, 55, and 80 °C). Figure 3 represents the MC morphological changes induced by solution heating. As compared to the AFM images shown in Figure 2a,b (4 °C), the MC chains formed fibrous bundles with flexible MC wire chains in the case of a solution temperature at 25 °C; the chain alignment became obscure and a partial chain assembly with a thickness of ca. 1–3 nm was found (Figure 3a,b). The solution temperature of 55 °C gave rise to both flexible wires and entangled aggregates of MC chains whose thickness was more than 3 nm (Figure 3c,d), while the higher temperature of 80 °C resulted in the formation of massive aggregates of MC chains (Figure 3e,f). As indicated in the series of AFM images (Figures 2a,b and 3), such conformational dynamics of the MC chains on the HOPG surface at different solution temperatures are consistent with the fundamental knowledge obtained from previous rheological studies: the MC molecular assembly formed via hydrophobic interactions in the solution state is accredited to the microphase separation of the heterogeneous MC chains with both hydrophobic MC and hydrophilic AHG units by heating (Figure 4).[1–6] The observed morphological changes at the single molecular level presumably support the proposed mechanisms of MC gelation; that is, the hydrophobic molecular association occurs and becomes stronger as the solution temperature increases, leading finally to massive MC aggregati

due to the formation of hydrophobic junctions (Figure 4). However, in fact, such a gelation phenomenon was not found in the case of the dilute MC solution of 10 mg L^{-1}, although a more concentrated solution (10 g L^{-1}) exhibited the reversible gelation feature as illustrated in Figure 1. Hence, there were certain differences between the AFM imaging and visual inspection and thus dynamic viscoelastic analysis was carried out in detail to assess the AFM results.

Rheological Behavior of Dilute MC Solutions. Figure 5 profiles the shear storage and loss moduli (G' and G'', respectively) curves of aqueous MC solutions as a function of frequency at various solution temperatures. The plateau G' and G'' profiles of a concentrated MC solution (10 g L^{-1}), as shown in Figure 5a,b, respectively, suggested that some sort of network structure of the MC polymers that can sustain stress was present in aqueous solution at the initial stage, and a drastic increase in the G' values from 60 to 70 °C presumably indicated that the distinct gel formation occurred around the LCST (ca. 65 °C).[3] On the other hand, almost no significant changes were detected in the rheological characteristics of the 10 mg L^{-1} MC solution because of huge data scattering as shown in Figure 5c,d; this was inconsistent with the AFM data (Figures 2a,b and 3). Hence, it seems to be possible to detect the molecular assembling behavior of MC polymers more sensitively by the AFM imaging than the rheological measurement. The concentration of the MC solution is one of the critical factors for the thermal responsibility. In the previous rheological studies, both the sol–gel transition and phase separation of the MC solution were investigated by using a concentrated MC solution (>10 g L^{-1}).[2–4] It has also been reported that it is difficult to discuss MC gelation based on the gradually increasing G' curves at low MC concentrations (<2.5 g L^{-1}) during the heating process.[3] These rheological studies provide essential information on the thermoresponsive assembly of MC chains; however, the MC association at the nanometer scale in dilute solution was poorly reflected in the rheological data. Therefore, the rheological behavior does not always give an account of the whole picture of the real MC association phenomena. From the viewpoint of the AFM imaging of MC chains via the momentary contact of a dilute MC solution onto an HOPG surface, it would be reasonable to consider that the MC molecules on the HOPG surface could reflect their conformation in the solution state. Even though the possibility of some negative impact of the MC/HOPG interaction must be further investigated, the AFM imaging of single MC chains demonstrated significant conformational changes dependent on the solution temperature. Thus, comparison of the rheological behavior with these AFM images implies that the delicate nanoassembly of MC chains at the molecular level may be insufficiently detectable by conventional rheological techniques. Therefore, successful visualization of MC nanomorphological dynamics on an HOPG substrate can compensate for shortcomings in viscoelastic analyses, and would provide direct visual information on MC molecular dynamics.

Conclusion

Single molecular MC chains were immobilized onto an HOPG substrate and clearly visualized by tapping-mode AFM analysis through the momentary contact of a dilute aqueous MC solution onto the substrate surface. Morphological changes in the MC chains at different solution temperatures were successfully observed as a snapshot of the solution-state MC molecules on the HOPG surface; the molecular assembly of MC chains was sensitively induced by increasing the solution temperature, and this information is poorly reflected in the rheological behavior. Clear visualization of individual MC molecules and their nanoassembly would provide fundamental and valuable information on the molecular features and material design of various structural polysaccharides.

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