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ORIGINAL CONTRIBUTION

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Conformation of curdlan as observed by tapping mode atomic force microscopy

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Abstract Tapping mode atomic force microscopy was used to study the bacterial polysaccharide curdlan deposited from dimethyl sulfoxide (Me_2SO) and NaOH aqueous solutions. For curdlan in Me_2SO, flexible single chains corresponding to a disordered conformation were observed at a concentration of 5 mg/l, and the chain diameter was measured to be 0.65±0.05 nm, which showed good agreement with the expected value of the single polysaccharide chain. Because the concentration of curdlan increased, the chains became more rigid and aggregated, subsequently, the network structures of curdlan appeared. However, curdlan samples deposited from a 5 mM NaOH solution showed entirely different conformations. The chains observed were almost in the form of micelles of several nanometers, which were supermolecular assemblies. The heterogeneously dense zones were observed as the curdlan concentration increased to 40 mg/l. When the concentration of curdlan was above 100 mg/l, which might cause the real concentration of curdlan on the mica substrate after drying treatment exceeding some critical value of gelation, gel network structures were formed. Keeping on increasing the concentration of curdlan, the image showed a more homogeneous fibrous network.

Keywords Curdlan · Tapping mode atomic force microscopy · Conformation · Dimethyl sulfoxide · Sodium hydroxide

Introduction

Curdlan is an extracellular polysaccharide produced from soil microorganism *Alcaligenes faecalis* var. 10C3K, and its linear structure is composed entirely of (1→3)-β-D-glucosidic linkages [1], as shown in Fig. 1. In the solid state, its molecules seem to exist as a triple helical structure [2, 3]. Curdlan is not soluble in water but can be dissolved in alkaline aqueous solution and dimethyl sulfoxide (Me_2SO). The water insolubility of curdlan may be attributed to the existence of extensive intra- and intermolecular hydrogen-bonded crystalline domains like that of cellulose [4]. The conformation of curdlan in alkaline solutions was studied extensively and it is reported that the conformation changes from a triple helix to a disorder random coil structure at around 0.2 M NaOH [5, 6]. Curdlan aqueous suspensions have unique gelling properties of forming either low-set gels or high-set gels depending on the heating temperatures [4, 7]. In addition, curdlan derivatives are believed to show strong bioactivities such as antitumor and anti-HIV [8, 9]. These bioactivities seem to be related to high-order structure of curdlan in the solution.

It is known that the secondary structure of a biopolymer, such as chain conformation, one of the most important factors of biomacromolecules, significantly affects and governs the characteristics and applications of biopolymers. As an important prerequisite to understanding the physicochemical properties of curdlan, conformational study is essential for the elucidation of its gelation and bioactivities, and for its further utilization.

Most of the previous works were conducted by traditional techniques such as thermal analysis [10],

X-ray diffraction [11], and rheology measurements [12]. Nowadays, atomic force microscopy (AFM) is widely used to study the molecular and supermolecular structures of biological macromolecules, and many successful applications of AFM to biopolymers were achieved [13–15]. Vuppu et al. [16] reported AFM images of curdlan in aqueous solution and scleroglucan in Me_2SO. Recently, Ikeda and Shishido [17] also studied the heat-induced gelation of curdlan by AFM. However, the systematic AFM studies of curdlan are still limited and there were no microscopic studies of curdlan deposited from Me_2SO solution. In the present work, by using tapping mode AFM (TmAFM), we described the molecular resolution images of the curdlan deposited from Me_2SO and from low concentration NaOH solutions in detail.

Experimental

Curdlan was given by Takeda-Kirin Foods Corporation (Tokyo, Japan) and was used without further purification. Curdlan powders were dissolved in reagent grade Me_2SO to make a 1-mg/ml stock solution under stirring for a few days. Samples of curdlan in Me_2SO were made by diluting the 1-mg/ml stock solution to the required concentrations and the diluted solutions were stirred and equilibrated for 24 h before AFM sample preparation. Samples for AFM observation were prepared using the drop deposition method: a 3-μl aliquot of the solution was pipetted directly onto a freshly cleaved mica surface. A similar procedure was used to make the samples of curdlan deposited from a 5-mM NaOH solution. The free surfaces of the samples were allowed to dry in air for 10 min in an enclosed petri dish. Then the samples deposited from the NaOH and the Me_2SO solutions were kept in a desiccator to be dried for more than 12 and 24 h before imaged, respectively.

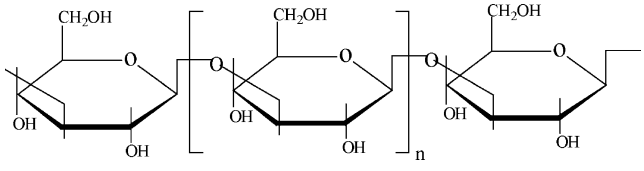

Fig. 1 Chemical structure of curdlan

AFM measurements

AFM observations were carried out using a NanoScope III AFM from Digital Instruments (Santa Barbara, CA, USA). Images were collected using tapping mode. A triangular 200-μm-long, 40-μm-wide cantilever with a pyramidal tip was adopted. The height information was recorded at a scan rate of 2 Hz and stored as 256×256 point arrays. All measurements were performed in air at ambient pressure and humidity (about 50% relative humidity).

Results and discussion

AFM observation of curdlan deposited from Me2SO solution

A typical TmAFM image for curdlan molecules deposited from Me_2SO solution onto the mica is shown in Fig. 2. To image an individual chain of curdlan, it is necessary to maintain its solution at a sufficiently low concentration so that the molecules are evenly separated from each other. In this study, we imaged the single molecules of curdlan at the concentration as low as approximately 5 mg/l. Generally speaking, the dispersed curdlan molecule in Me_2SO is expected to be a flexible coil because the Me_2SO molecules are capable of cleaving the intra- and intermolecular hydrogen bonding [18]. Compared with the conformation of pullulan in aqueous solution [19], the curdlan chain in Fig. 2 should not be considered as a random coil but a slightly stiffened coil chain.

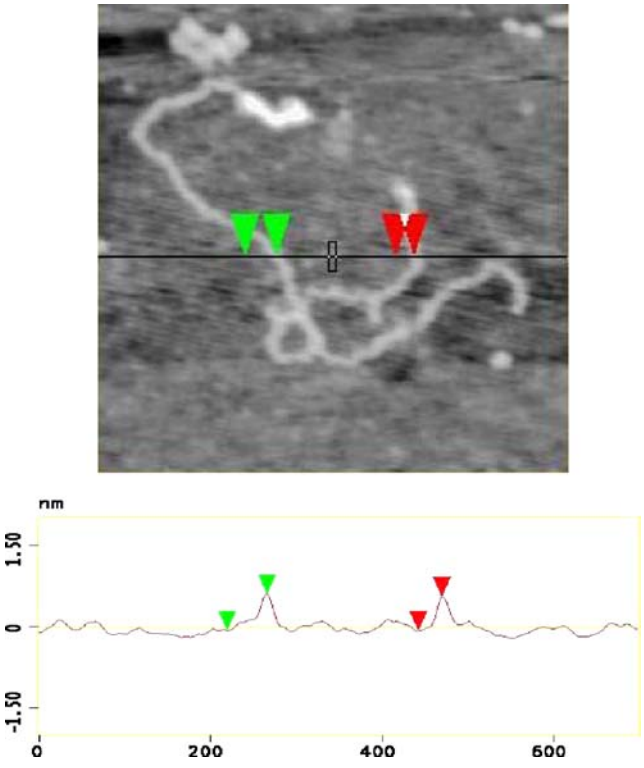

Fig. 2 TmAFM image of curdlan deposited onto mica surface. Image was obtained by depositing curdlan at a concentration of 5 mg/l in Me_2SO. Image size: 700×700 nm. The *curve* beneath the image represents the height profile of the cross-section highlighted in the image

Compared with the AFM results obtained by Vuppu [16] who observed the molecular conformation of scleroglucan in Me_2SO, which differs from our curdlan only in the presence of the 1- to 6-linked glucopyranosyl side group, the images were absolutely different. For scleroglucan, the random coils approached each other and formed amorphous aggregates that either carpet the mica substrate or create spherical structures to minimize surface forces. The difference in conformation might originate from two aspects: firstly, maybe the concentration they used was still too high that the molecules on the mica could not be detected in the form of single flexible chain and secondly, because scleroglucan could be dissolved in water, during drying in air before being imaged in 4 days, perhaps the conformation on the mica renatured from a single chain to triple helices due to hydration.

Tracing across individual curdlan strands provided an estimate of the height of the macromolecule corresponding to its diameter. The height value of the individual curdlan strand in Fig. 2 was measured to be 0.65±0.05 nm, which is in good agreement with the expected diameter of a single polysaccharide chain based on the model-building studies [20]. We considered this value to be the height of the single chain of curdlan. Nonetheless, some discrepancy may exist between this value and that determined by crystallography. McIntire and Brant [21] found that for scleroglucan, the height measured by AFM was approximately 67% of the thickness expected from X-ray fiber diffraction. They thought that the dissimilarity might result from the fact that the molecules were distorted by desiccation or by interaction with the mica substrate, or that the molecules were partially embedded in a layer of water adhering to the mica surface.

As the concentration of curdlan increased to 40 mg/l, the chains dispersed on the mica were not symmetrical as shown in Fig. 3 in which both individual chains and their aggregates were observed. The aggregation of curdlan may be expected due to the formation of the inter- and intrahydrogen bonding in the solution. For a curdlan–Me_2SO system, OH groups only come from curdlan chains. It is likely that apart from self-association of curdlan, the association of macromolecules involving Me_2SO contributed to the formation of aggregation. In addition, the poor affinity of curdlan for the mica substrate also resulted in the formation of heterogeneous zones, indicating that the polymer was likely displaced during the drying process. On the mica surface in low concentration areas in Fig. 3, curdlan exhibited as a single chain with the same diameter as detected in Fig. 2, implying that the aggregates are ensembles of single chains.

In addition, as the concentration increased, the number of curdlan molecules in which hydrogen bonding was cleaved by Me_2SO decreased, and more molecular chains can hold their intrinsic stiff helical structure. As a result, the flexibility of the chains decreased markedly and adopted a more rigid chain.

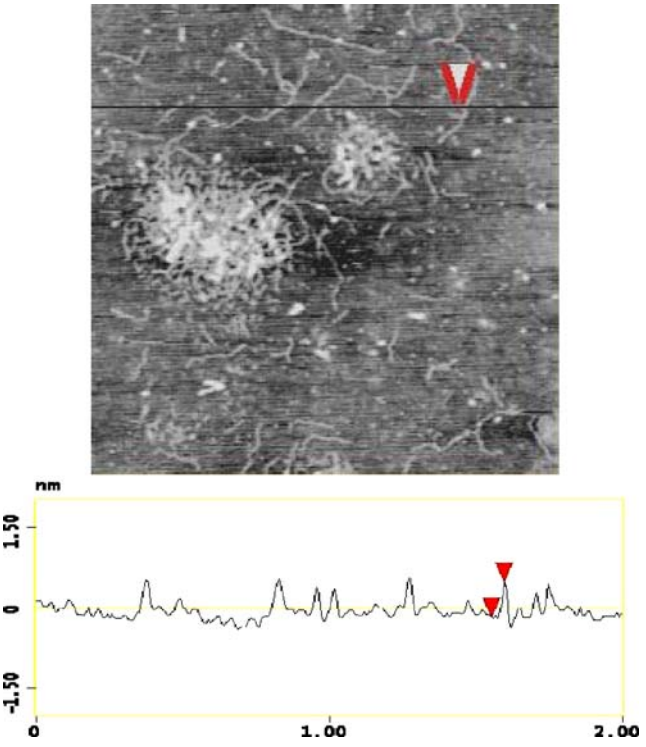

Fig. 3 TmAFM image of curdlan deposited onto mica surface. Image was obtained by depositing curdlan at a concentration of 40 mg/l in Me_2SO. Image size: 2.0×2.0 μm. The *curve* beneath the image represents the height profile of the cross-section highlighted in the image

When the concentration increased to about 100 mg/l, curdlan in Me_2SO was capable of forming a web-like layer as shown in Fig. 4. It may be due to both curdlan self-association and bridging association involving Me_2SO through hydrogen bonding. The image was quite different from that at a low concentration in the point that many chains aggregated each other tightly, forming continuous network structures. The image is consistent with the rheological results in which the storage modulus of curdlan Me_2SO solution at a higher concentration is larger than loss modulus due to curdlan molecules forming entangled networks in Me_2SO [18].

The images of curdlan deposited from Me_2SO solution changed from an individual chain to the network structures with the increase of concentration. To our knowledge, these images we obtained were the first to characterize a single chain and the morphology of curdlan deposited from Me_2SO.

AFM observation of curdlan deposited from a 5-mM NaOH solution

Unlike Me_2SO that is a pure solvent, the other solvent for curdlan, alkaline aqueous solution, is used to acquire curdlan images. In our experiment, it was found that the

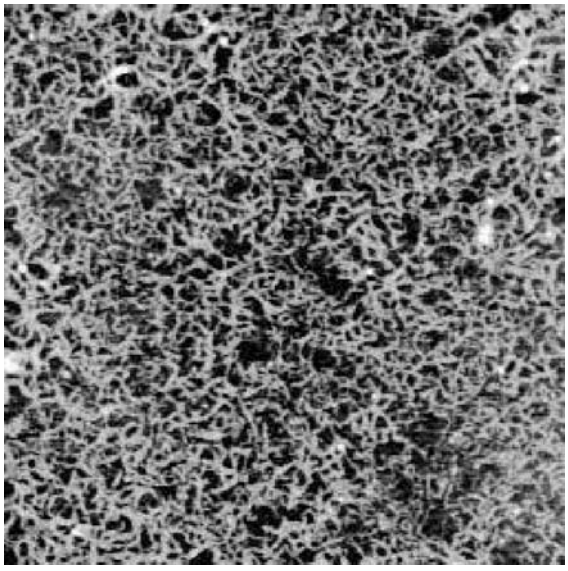

Fig. 4 TmAFM image of curdlan deposited onto mica surface. Image was obtained by depositing curdlan at a concentration of 100 mg/l in Me_2SO. Image size: 2.0×2.0 μm

alkaline concentrations above 0.02 M were not suitable for observing the curdlan molecules because the precipitation of alkaline solids influenced the molecular conformation during drying treatment, and the degree was proportional to the alkaline concentration used. For our present purpose, it was not possible and was meaningless to get the AFM images at too high alkaline concentration. Fulton and Atkins [22] proposed that triple helices were dominant for most curdlan molecular chains, and the triple strand molecules were bound by hydrogen bonding with the interstitial water to form a micellar domain. It was reported that for curdlan, the dissociation occurred in NaOH solution of at least 0.05 M [3]. So when the weak alkaline solution was used, the curdlan micelles could almost be maintained and could not be dissociated. Thus, an alkaline concentration of 5 mM was chosen. In such a case, curdlan might be of an analogous structure to that in solid state [23].

A previous AFM observation for curdlan deposited from a 5-mM NaOH solution at a concentration of 10 mg/l shown in Fig. 5 revealed that the multiple curdlan molecules are almost in the form of thick, rope-like structures that are considered as micelles [23]. Furthermore, the triple helices with a diameter of about 1.34 nm were also obviously observed as shown in Fig. 5b. This value of the diameter is approximately 86% of the triple strand thickness (1.56 nm) detected from X-ray fiber diffraction [11]. The discrepancy in magnitude of the diameter of curdlan triple helices might be because triple helices were distorted by interactions with the mica substrate or partially embedded in a layer of water adhering to the mica surface. As far as the contour length is concerned, the contour length for curdlan in 5 mM of NaOH is much longer than that for curdlan in Me_2SO. This fact supports that the observed micelles in NaOH solution are composed of associated bundles of triple helices.

The observed height of these micelles in Fig. 5 is about 6.5 nm, which is quite higher than those obtained by Ikeda and Shishido [17] for samples prepared at 600 mg/l in 0.01 M NaOH or 200 mg/l in 1 M NaOH. For AFM samples, despite dehydration in air, the surfaces of micelles in Fig. 5 should be surrounded with a hydrated layer, which

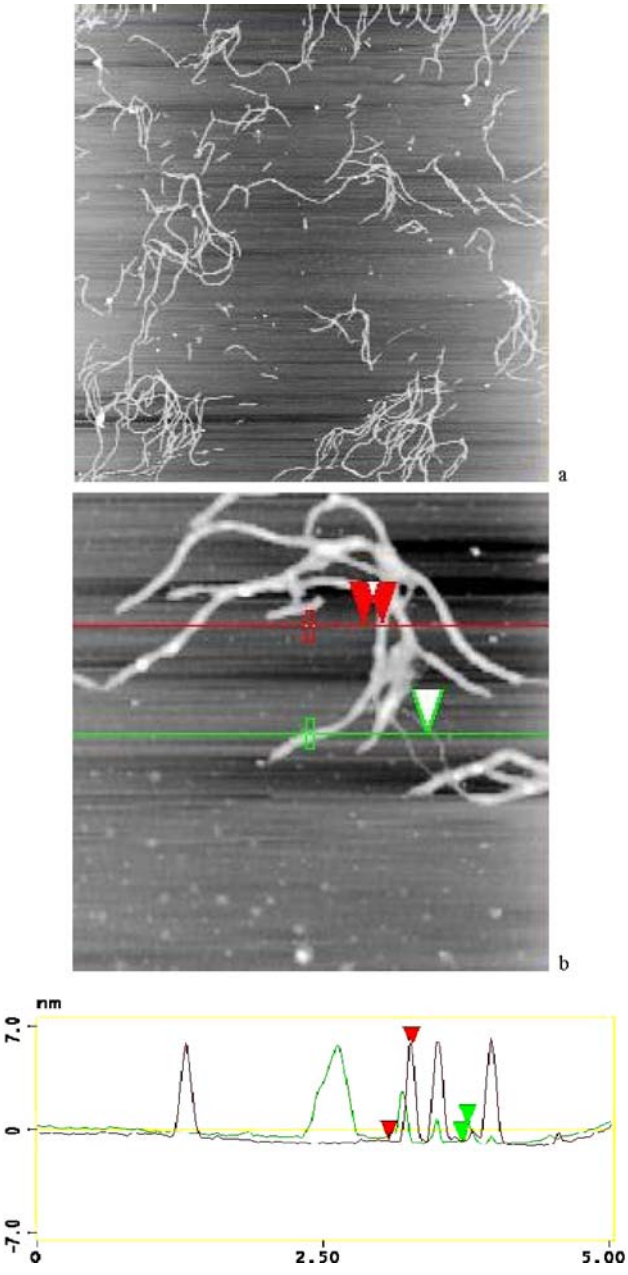

Fig. 5 TmAFM images of curdlan (10 mg/l dissolved in 5 mM of NaOH) deposited onto mica surface. Image size: **a** 20.0×20.0 μm and **b** 5.0×5.0 μm. The *curve* beneath the image represents the height profile of the cross-section highlighted in the image

would result in the decrease of the height measured. The observed heights of the chains decreased with increasing degree of hydration so the observed values of height for the micelles might be underestimated. Sometimes the underestimated height of the triple helix might be considered as much as that of the single chain. In the present work, the diameter of the micelle is roughly equal to 8 nm, being consistent with the estimated value based on X-ray small angle scattering [24].

As the curdlan concentration increased to 40 mg/l, AFM image revealed that in some areas on the mica surface the macromolecules were present as individual micelles while in some other areas the micelles were very concentrated and were overlapping as seen in Fig. 6. The poor affinity of curdlan for the mica substrate resulted in the observation of heterogeneous zones, indicating that the polymer was most likely displaced during the drying process. The height of the dispersed aggregated micelles is about 7.00±0.50 nm, similar to that at lower curdlan concentration in Fig. 5.

Figure 7 shows AFM images of curdlan network formed by depositing a 100-mg/l curdlan solution onto mica. Because of the uneven distribution of the concentration on the mica, the image in Fig. 7a shows a heterogeneous network structure. The method developed for imaging individual micelles by AFM can be extended to image the polysaccharide network. When the concentration of curdlan exceeds some critical value for gelation, the gel structures are formed on the mica surface. In spite of the extremely low concentration of curdlan when deposited on the mica surface, it is reasonable to expect that the concentration will increase remarkably up to or beyond the critical gelling one during the drying treatment although the exact final concentration is difficult to be estimated. The gel structures in Fig. 7 exhibited a repeating network of solvent cavities caused by the evaporation of solvent and curdlan strands. The measured thickness of these strands in the gel matrix ranged from 4.20 to 7.13 nm. The thickness of the strands is much larger than those estimated for individual curdlan molecules, consistent with the thickness

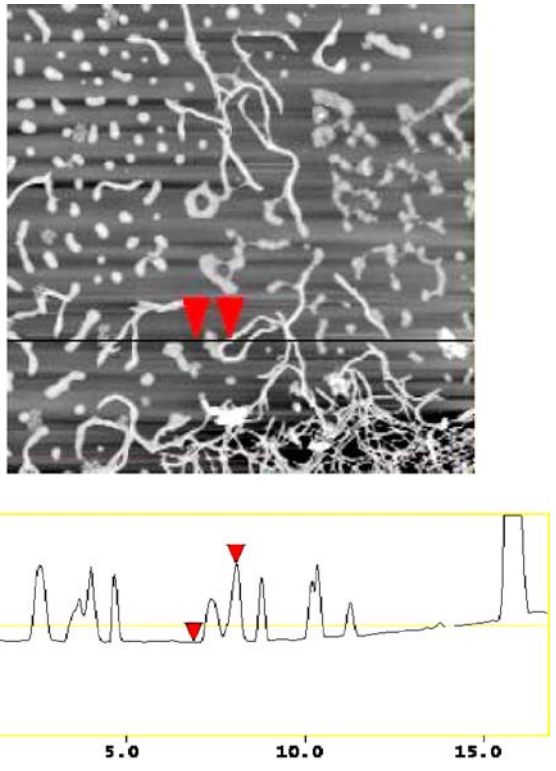

Fig. 6 TmAFM image of curdlan (40 mg/l dissolved in 5 mM of NaOH) deposited onto mica surface. Image size: 15.0×15.0 μm. The *curve* beneath the image represents the height profile of the cross-section highlighted in the image

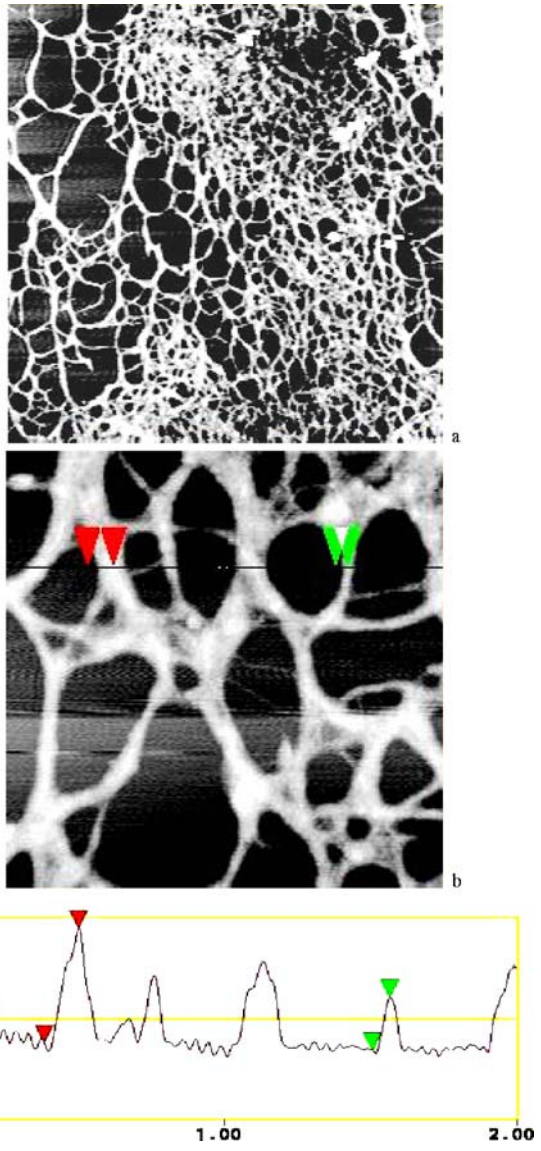

Fig. 7 TmAFM images of curdlan (100 mg/l dissolved in 5 mM of NaOH) deposited onto mica surface. Image size: **a** 10.0×10.0 μm and **b** 2.0×2.0 μm. The *curve* beneath the image represents the height profile of the cross-section highlighted in the image

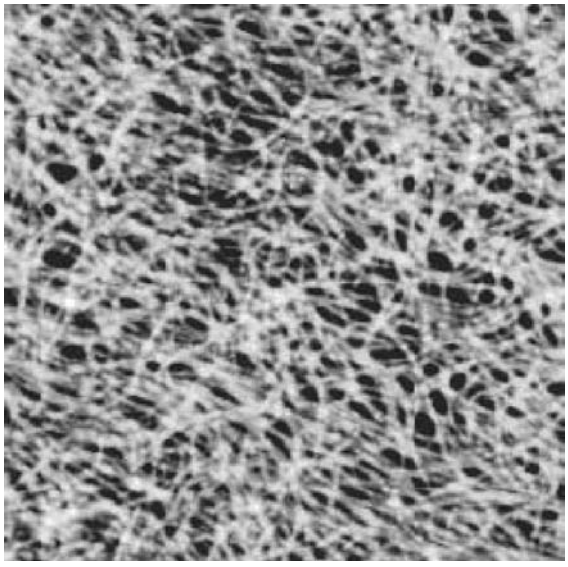

Fig. 8 TmAFM image of curdlan (150 mg/l dissolved in 5 mM of NaOH) deposited onto mica surface. Image size: 5.0×5.0 μm

of the micelles as mentioned above, suggesting that the gel network is not formed by individual chains, but formed by the micelles. In comparison with the height distribution of micelles in Figs. 5 and 6, the decrease in height for some micelles might be attributed to the surrounding water layer. The hydration degree for curdlan at higher concentration is most likely stronger than that in the case of lower concentration.

The reasons that the gel network of curdlan can form on mica surface from the air-dried sols are related to several aspects. Firstly, it is related to the concentration of curdlan in solution and the change in concentration on the mica surface during the drying process. On the one hand, it is found that for the original samples, the concentration below 60 mg/l is too low to permit gelation. On the other hand, when the initial concentration of curdlan is beyond 60 mg/l, the gelation of curdlan begins to be favored by the increasing of the concentration of curdlan due to the water evaporation on substrate surface. And secondly, curdlan gels can be formed by various methods, one of which is neutralizing an alkaline solution. Considering the low alkalinity of the solvent used (pH 11.7), the carbon dioxide in the air might neutralize the NaOH solution on the mica during the sample preparation, subsequently resulting in the occurrence of gelation.

In addition, it is also found that when the curdlan concentration is 150 mg/l, the effect of the mica substrate on the molecules becomes weak and the images of network become more regular and homogeneous as shown in Fig. 8. However, if the curdlan concentration is above 200 mg/l, the network of curdlan on the mica surface is too dense to be identified by AFM.

Conclusions

The present study demonstrated the utility of TmAFM for observing individual chains, aggregates, and gel network structure of curdlan in Me_2SO and 5 mM of NaOH solution. Molecular conformations determined by AFM were shown to be affected by local concentration of the polysaccharide. In DMSO, curdlan existed in the formation of a single chain, whereas in weak alkaline solution, the curdlan molecules were almost in the form of micelles composed of some triple helices.

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