Conformations of Succinoglycan As Observed by Atomic Force Microscopy

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ABSTRACT: Succinoglycan, a high molar mass polysaccharide, undergoes conformational transformations as a function of ionic strength. The nature of the transitions and the presence of intermolecular associations have been described previously using solution-based techniques. In this work, we have determined the conformation of succinoglycan macromolecules at the solution−mica interface using atomic force microscopy (AFM) and compared these data to the measurements obtained in solution. Molecular characteristics such as chain length, end-to-end distances, polymer heights (diameters), and chain rigidity were determined as a function of ionic strength. Individual chains and dimers were found for succinoglycan deposited from pure water, whereas only individual chains were found for 0.01 M KCl. In 0.5 M KCl, succinoglycan formed a gel-like structure at the mica surface. Analysis of persistence lengths from the AFM images indicated that succinoglycan became more rigid with increasing ionic strength. Flexible chains corresponding to a disordered conformation were observed in water while ordered, single helical chains were imaged in 0.01 M KCl. In comparison to bulk solution measurements, molecular conformations determined by AFM were shown to be affected by local concentration increases due to the AFM drying step and by the strength of the interaction between the macromolecules and the mica substrate. In water and 0.01 M KCl, comparison of the measured end-to-end distances, with calculated 2D or projected end-to-end distances, revealed that the polysaccharide was not at equilibrium with the mica surface. These findings demonstrate the potential of AFM as a polymer characterization technique that is complementary to classical solution-based techniques and able to provide specific information on the polymer conformations at the solid−water interface.

Introduction

Succinoglycan is an anionic bacterial polysaccharide (Figure 1) which is known to undergo conformational transitions in solution as a function of the temperature and the ionic strength of the medium.[1−5] For example, most studies have indicated that succinoglycan is a single extended chain in aqueous solutions in absence of salt [e.g., refs 1 and 2], while a transition from a stretched to a single helical chain has been observed with increasing ionic strength.[3,6]

Intermolecular associations among the succinoglycan macromolecules are also possible. For example, dimers have been postulated to form in salt solutions through the association of the single helices due to a reduction in the electrostatic repulsion between the polysaccharide chains.[2] A similar association has been observed by Borsali et al.[6] using light scattering and small-angle neutron scattering techniques. They determined that while the polysaccharide was a single chain at low polymer concentrations (<50 mg L^{-1}), its organization into dimers was observed at concentrations >100 mg L^{-1}.

The existence and stability of the intermolecular associations will depend on both polymer properties such as molecular weight and the physicochemistry of the solutions, e.g., polymer and salt concentration and

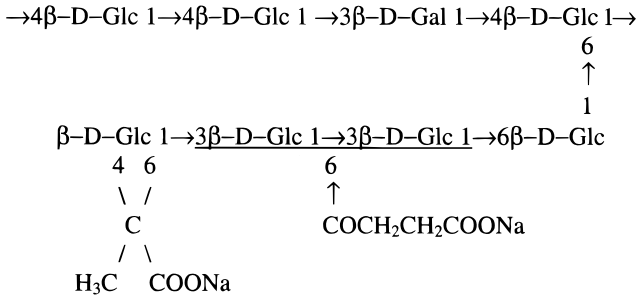

Figure 1. Chemical structure of succinoglycan.

temperature of the solution with respect to the transition temperature, T_m.[7,8] For example, Dentini et al.[1] have demonstrated that heating above the T_m followed by cooling below the T_m destroys the intermolecular associations, if initially present. Although the coil-to-helix transition favors intermolecular associations,[8] the light scattering data of Dentini et al.[1] suggest that associations of succinoglycan macromolecules including aggregates may occur in both water and salt solutions even at very low polymer concentrations.

Much of the difficulty in characterizing succinoglycan results from the fact that it may easily form aggregates in solution and that most of the techniques used to determine its conformation are statistical techniques which give an average response based on a large number of molecules. Atomic force microscopy (AFM) is an emerging technique which is able to provide direct

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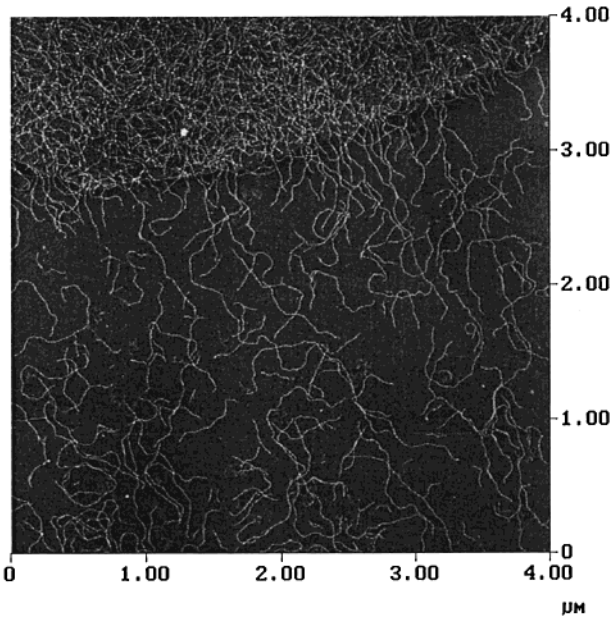

Figure 2. Height mode AFM images of succinoglycan (10 mg L^{-1}, dissolved in water) on mica showing heterogeneous zones of "rigid" chains (RC); scan size is 4 μm × 4 μm.

images of individual biomolecules with a nanometric resolution. Indeed, several polysaccharides have already been imaged by AFM, including xanthan,[9−11] schizophyllan and scleroglucan,[12,13] hyaluronan,[14] gellan,[15] and natural freshwater polysaccharides.[16] Nonetheless, it remains to be demonstrated unambiguously whether AFM, especially when operated in air, can provide statistically representative images of biopolymers at interfaces and whether the adsorbed biopolymer conformations are related to what is observed in solution. Until recently, only DNA has been thoroughly studied in parallel in solution and using AFM. For example, several groups[17,18] have demonstrated that the conformation of DNA in solution resembled that observed by AFM under ambient conditions (air-dried). Margeat et al.[18] used a statistical chain analysis to show that DNA molecules reached 2D conformational equilibrium prior to being trapped by the mica surface. Finally, our previous work examining natural aquatic biopolymers[19,20] suggested that while conformations may change following adsorption at interfaces, careful preparation of the sample could ensure that the information obtained by AFM pertaining to intermolecular associations was representative of that obtained in solution.

Therefore, the goal of the present study was to use AFM, operated under ambient conditions, to provide information on the conformation of succinoglycan on mica. From the AFM images of succinoglycan, molecular dimensions and variations in the molecular bending angles were determined. The effects of the ionic strength on inter- and intramolecular associations of the polymer and on polymer rigidity were deduced and compared with solution-based measurements.

Experimental Section

Preparation of Succinoglycan Solution. Two hundred mg L^{-1} of previously sonicated succinoglycan, produced and purified as described by Boutebba et al.,[8] was dissolved by mixing in water and gently stirring for at least 12 h at room temperature. The resulting solution was filtered through a 0.2 μm Nuclepore filter and then diluted to 10 mg L^{-1} in water, 0.01 M KCl, or 0.5 M KCl. The first two solutions were heated to 85 °C for 10 min in order to dissociate aggregated materials prior to a second filtration through a 0.2 μm Nuclepore filter. The weight-average molecular weight of the sample, as determined by multidetection size exclusion chromatography, was 1×10^6 with a polydispersity index of 1.4.

Sample Preparation for AFM. Samples were prepared using the drop deposition method. Some of the advantages and limitations of this technique have been discussed in detail elsewhere.[16] A 5 μL aliquot of 10 mg L^{-1} succinoglycan solution was pipetted onto freshly cleaved mica. The surface was allowed to dry for 30 min in an enclosed Petri dish prior to AFM observation. AFM images were obtained with a Nanoscope III multimode microscope from Digital Instruments (Santa Barbara, CA). Images were recorded in air at a relative humidity of 50% using tapping mode atomic force microscopy (TM-AFM).[21] Under these conditions, imaging is not completely "in air" since the mica retains a thin film of water roughly equivalent to the diameter of the macromolecules (0.25−1 nm).[22] To minimize the forces of interaction between the tip and the surface, the ratio of the set point amplitude to the free amplitude was maintained at 0.9.

Quantitative Analysis of the AFM Images. AFM images were digitalized using Scion image analysis software (Vbeta3b, based on NIH image software, Scion Corporation). The absolute total length and coordinates of each point along the chains were obtained using Sigma Scan Pro image analysis (Jandel Scientific). The contour length, L_{tot}, and the distance between the two extremities of the chain (the end-to-end distance, R_{ee}) were determined directly using the software. The persistence length (L_p) of the polysaccharide was determined from the reciprocal slope of a plot of the mean-square angular dependence, $\langle\theta^2\rangle$, against the macromolecular contour distance, l (eq 1).[23]

$$\langle\theta^2\rangle = \frac{l}{L_p} \tag{1}$$

In brief, the method that we used consists of measuring the angle, θ, between two successive coordinates of a series of approximately equidistant points along the polymer chain. For large distances between the points, the finite length of the molecules limits the number of possible independent choices of points so that a meaningful estimate of L_p is only obtained for the initial slope of eq 1.

The value of L_p will reflect the persistence length in solution if[23] (i) the observed two-dimensional conformation is not a projection of the 3D conformation of the biopolymer in solution, but instead results from an allowed deformation of the macromolecule on the surface, and (ii) the interaction of the polymer with the surface does not alter the local rigidity of the macromolecules. Under these conditions, information concerning the chain rigidity should be conserved; i.e., the conformation of the adsorbed polymer should be similar to that obtained in solution, and the distribution of angles along the polymer chain should be Gaussian. Verification that the ratio $\langle\theta^4\rangle/\langle\theta^2\rangle^2 = 3$ provides a test of the angular Gaussian distribution.[23]

The error on the measurements of the end-to-end and contour length distances was estimated to be about ±2 nm due to the resolution of the digitalized AFM images. For each condition, analysis was performed on 50 isolated macromolecules.

Results and Discussion

This section is organized into two parts: in the first, descriptions of the conformation of the succinoglycan and its characteristics (contour length, end-to-end distance and height for the different entities) based on the AFM images are presented for the different solution conditions. In the second part, the effects of ionic strength and the role of the substrate (mica) on the conformation of the macromolecules are discussed. To simplify the reading of the paper, molecular species are

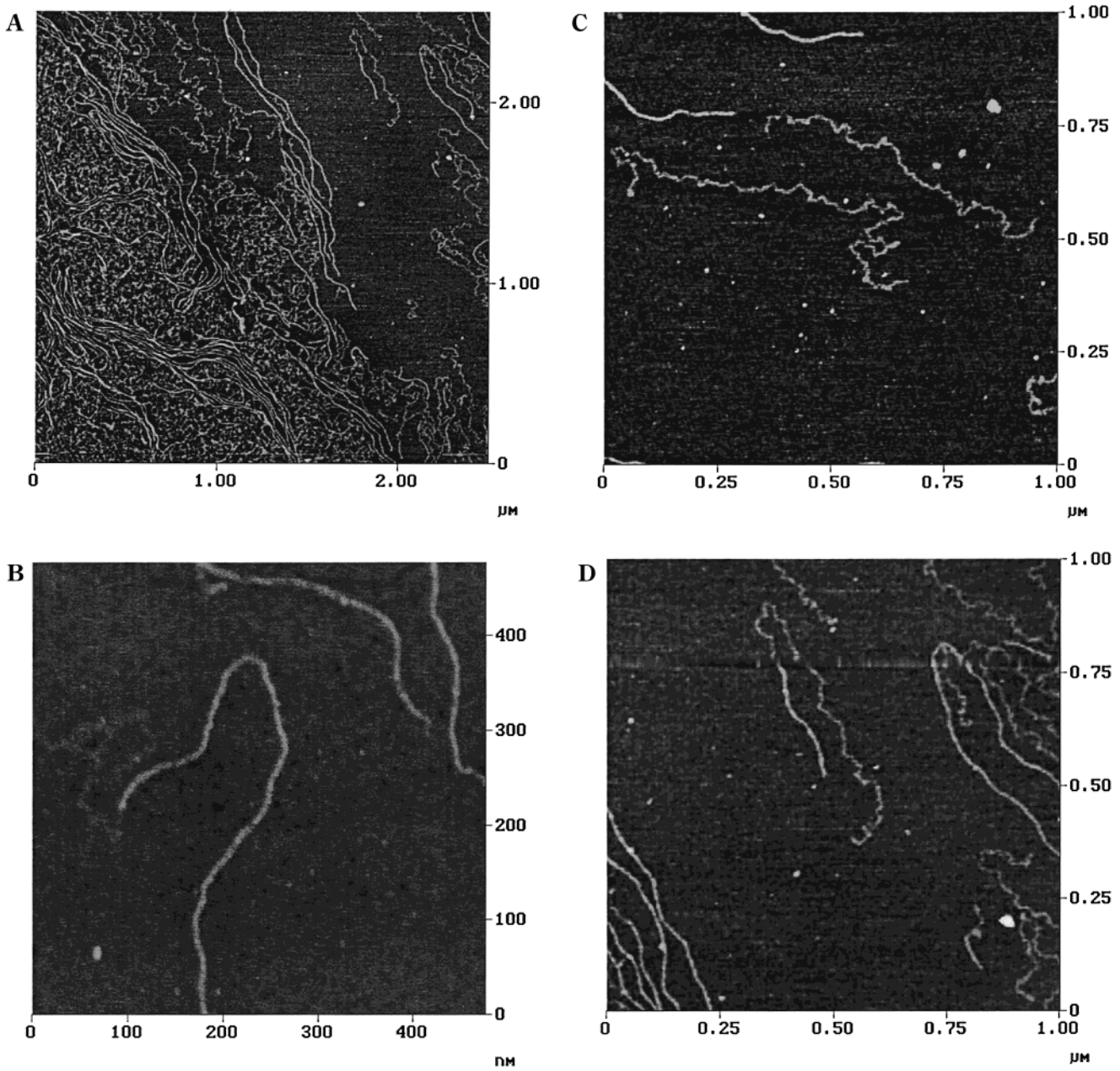

Figure 3. AFM images of succinoglycan (10 mg L^{-1}, dissolved in water) deposited on mica: (A) height mode image showing the coexistence of "rigid" and "flexible" chains, scan size is 2.5 μm × 2.5 μm; (B) "rigid" chain (RC), scan size is 500 nm × 500 nm; (C) "flexible" chain (FC), scan size is 1.0 μm × 1.0 μm; (D) magnified image of a molecule from (A) showing a rigid chain terminated by a flexible chain, scan size is 1.0 μm × 1.0 μm.

Table 1. AFM Characterization of the Different Succinoglycan Chains; Except Where Indicated by an Asterisk, All Values within a Column Are Significantly Different at $P < 0.05$ (One Way ANOVA; Student *t*-Test)[a]

	contour length, $\langle L_{tot} \rangle$	end-to-end distance, $\langle R_{ee} \rangle$	persistence length, $\langle L_p \rangle$	height $\langle z \rangle$
rigid chains in water	770 ± 460*	496 ± 257	105 ± 21	0.64 ± 0.05
flexible chains in water	704 ± 279*	297 ± 120	19 ± 7	0.32 ± 0.05
flexible chains in 0.01 M KCl	563 ± 278	178 ± 100	36 ± 13	0.44 ± 0.09

[a] All values in nm.

identified throughout as "flexible" or "rigid" chains even though the quantitative justification for this nomenclature is only given in the second section (determination of persistence lengths).

AFM Observation of Succinoglycan in the Absence of Salt. Although attempts were made to adsorb the polymer to the mica surface (adsorption method[16]), succinoglycan was not observed by TM-AFM following this method of sample preparation. This likely indicates that the electrostatic repulsion due the negative charges of the mica[24,25] and those of the succinoglycan (1 pyruvate and 0.75 succinate groups for each repeating unit; Figure 1)[6] overwhelmed the adsorptive forces under these experimental conditions (pH 5.8). On the other hand, the use of the drop deposition technique, which forces the polymer onto the mica surface, allowed the imaging of single succinoglycan chains. However, in this case, the poor affinity of the polymer for the

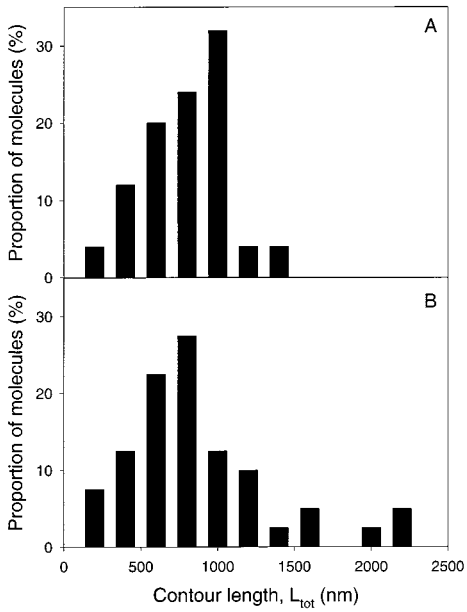

Figure 4. Histogram of the contour lengths of succinoglycan chains in water: (A) "flexible" chains; (B) "rigid" chains.

substrate resulted in the observation of heterogeneous zones (Figures 2 and 3A), indicating that the polymer was likely displaced during the drying procedure. AFM images revealed that in some areas the macromolecules were present as individual chains while in others they were very concentrated, at times overlapping. Despite this heterogeneous distribution of chains, two main types of structures were observed: rigid chains (RC) (Figure 3B) and flexible chains (FC) (Figure 3C). The mean height of the flexible chains was 0.32 ± 0.05 nm, which corresponds well to the thickness of a single chain, i.e., the width of a glucose unit is about 0.3 nm. The number-average contour length of the flexible chains on the mica surface was 704 ± 279 nm (Figure 4A), while the number-average end-to-end distance was 297 ± 120 nm. It should be noted that while the large standard deviations are due in part to the small number of macromolecules that were measured ($N = 50$), they mainly reflect the polydispersity of the polysaccharide. Assuming a length normalized molar mass of 750 g mol^{-1} nm^{-1} for a single chain of succinoglycan,[3] the estimated number mean molar mass obtained from the AFM images of the flexible single chains would correspond to about ~528 000 g/mol, in reasonable agreement with the M_n of 714 000 g/mol measured by size exclusion chromatography (SEC), especially considering that aggregates are ignored in the AFM calculations but not in the SEC determinations.

Rigid chains appeared less convoluted than the flexible chains (cf. Figure 3B,C). In this case, the mean height of the chains was 0.64 ± 0.11 nm, the measured contour length was 770 ± 460 nm (Figure 4B), and the mean end-to-end distance was 496 ± 190 nm. The increased mean diameter (height) of the rigid chain (i.e., 0.32 vs 0.64 nm; Figure 5) suggests that the structure is due to the association of at least two individual chains (dimer). Indeed, in some cases (e.g., Figure 3D), chains were observed which appeared both rigid and flexible. These qualitative observations were supported by the important height differences observed along the chain ("flexible" portion: 0.33 nm; "rigid" portion: 0.56 nm; Figure 3D).

Although these observations contrast with the measurement of disordered succinoglycan chains made at this concentration in solution,[3] the organization of the succinoglycan chains into dimers has also been observed by Borsali et al.,[6] albeit for higher polymer concentrations (>100 mg L^{-1}) than the 10 mg L^{-1} used here. In this light, it cannot be discounted that the presence of dimers in the AFM images might be due to the drying step used prior to the AFM imaging that could have locally increased the polymer concentration. Such an increase may have resulted in an increase of the T_m, a coil-to-helix transition, and a corresponding increased association between single chains.[3] The similar values of contour lengths concurrent with an average measured height of the rigid chains which is double that of the flexible ones is consistent with the fact that the rigid species are formed from a dimerization of the flexible chains.

AFM Observations in 0.01 M KCl. In 0.01 M KCl, succinoglycan macromolecules appeared primarily as individual chains but also included some macromolecular aggregates (Figure 6). In contrast with the AFM images of succinoglycan in water, the observation of a second polymer population was not made in 0.01 M KCl. Furthermore, heterogeneous zones were not observed, as was the case in water. In 0.01 M KCl, charge repulsion between the mica and succinoglycan is expected to be less important than in water due to screening effects. The number-average contour length of the succinoglycan macromolecules was 563 ± 278 nm (Figure 7), and the mean end-to-end distance was 178 ± 100 nm. Although the somewhat lower contour lengths in KCl as compared to water is consistent with a conformational transition from a disordered flexible chain in water to an ordered helical structure in salt, the interpretation must be made cautiously, given the large polydispersity and small sample size reflected in the relatively large standard deviations of the measurements.

In 0.01 M KCl, the measured heights of the chains were 0.44 ± 0.09 nm, intermediate to the values measured for the flexible and rigid chains in water (Table 1). While the AFM height increase (0.44 nm vs 0.32 nm for the flexible chains) is also in agreement with the presence of an ordered helix in 0.01 M KCl, caution must be applied when comparing AFM height measurements in different media. For example, Müller and Engel[26] observed height anomalies as a function of ionic strength using contact mode AFM. In their work, they were able to determine accurate height measurements of purple membranes, with respect to crystallographic data, by adjusting the electrolyte concentration so as to eliminate the double-layer forces between the AFM tip and the mica surface. In our case, the system is even more complicated because, in the presence of salt, not only will the tip−mica repulsive forces be reduced, but the tip−polymer interactions will also be modified simultaneously. It is therefore difficult to compare height measurements from one medium to another especially in the difficult-to-calibrate nanometer size range. In summary, although the data in 0.01 M KCl are consistent with data obtained in solution indicating the formation of a simple helical structure, interpretation of the AFM data must be made with great care.

AFM Observations in 0.5 M KCl. In 0.5 M KCl, the macromolecules were observed to form a gel-like structure on the surface of the mica (Figure 8A,B). In these

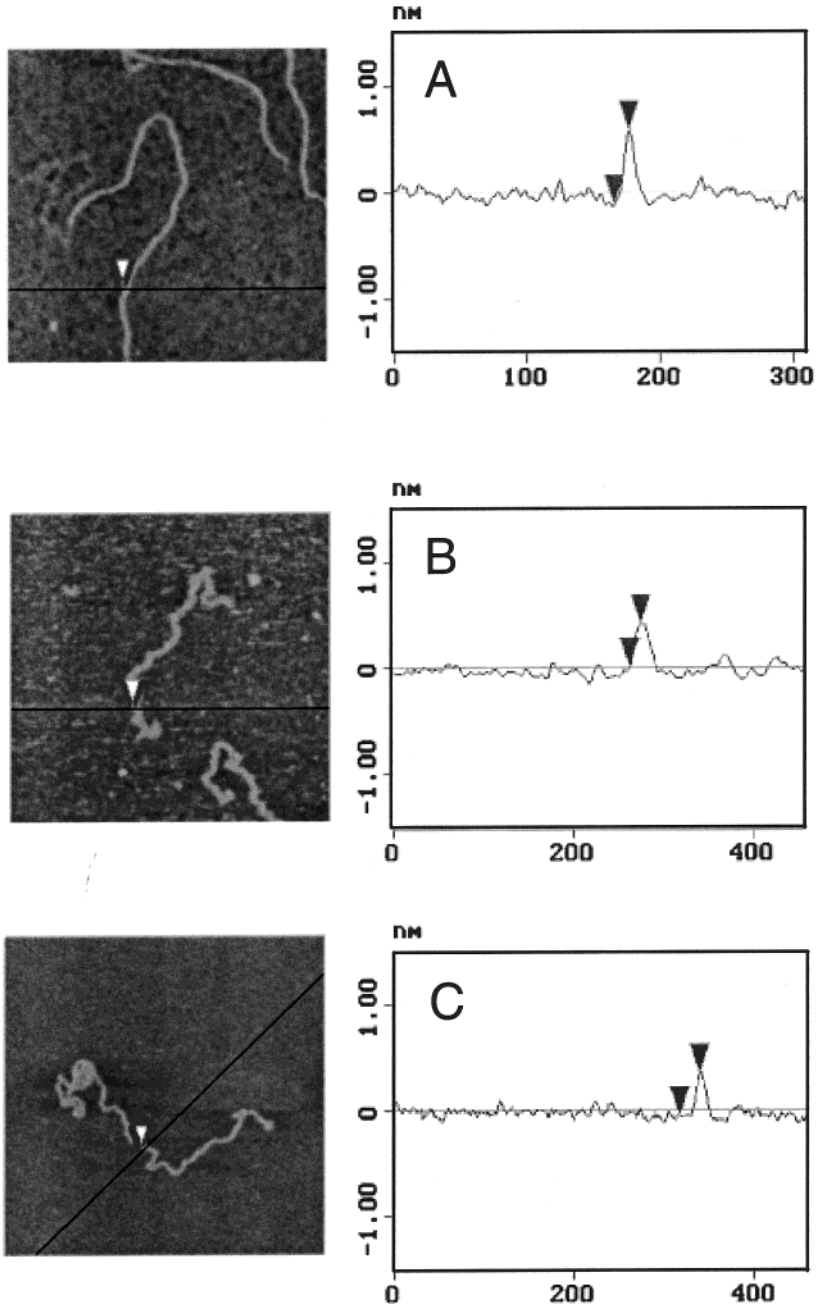

Figure 5. Section analysis of AFM images of the three different types of succinoglycan chains: (A) "rigid" succinoglycan chain in water, height = 0.69 nm; (B) "flexible" succinoglycan chain in water, height = 0.41 nm; (C) "flexible" succinoglycan chain in 0.01 M KCl, height = 0.40 nm.

images, the height of the branches varied between 0.6 and 1.2 nm, indicating that the gel network was not formed with individual chains, but with aggregates of the succinoglycan chains. These images are in contrast with the observations which were made in water or in 0.01 M KCl. In those cases, even in the absence of filtration, no evidence of gel formation was observed. On the basis of the tendency of high concentrations of succinoglycan to form gels in high ionic strength solution,[8] the AFM observation of a gel is in agreement with predicted results.

Persistence Length Determinations. The mean-square angle between points on the chains, $\langle\theta^2\rangle$, was plotted as a function of the contour distance of the macromolecules, l, for each of the three populations (Figure 9). In the absence of electrolyte, the persistence length, L_p, calculated from the inverse of the slope of the linear portion of the curve (eq 1), was 105 ± 21 nm for the rigid chains and 19 ± 7 nm for the flexible chains. The literature value of L_p in water of 5 nm[27] is smaller than the AFM determined value of 19 ± 7, suggesting that the molecule may have undergone conformational changes following its deposition on the mica surface.

In 0.01 M KCl, the L_p for the flexible chains was estimated to be 36 ± 13 nm, which was significantly different from L_p values for the flexible chains in pure water (Student t-test, $P < 0.05$). This result indicated that the succinoglycan macromolecules became more rigid at higher ionic strength, similar to what has been observed by bulk measurements in solution.[28,29] These observations are also in reasonable agreement with those of Morfin et al.,[30] who reported that the persistence length of succinoglycan was smaller in its

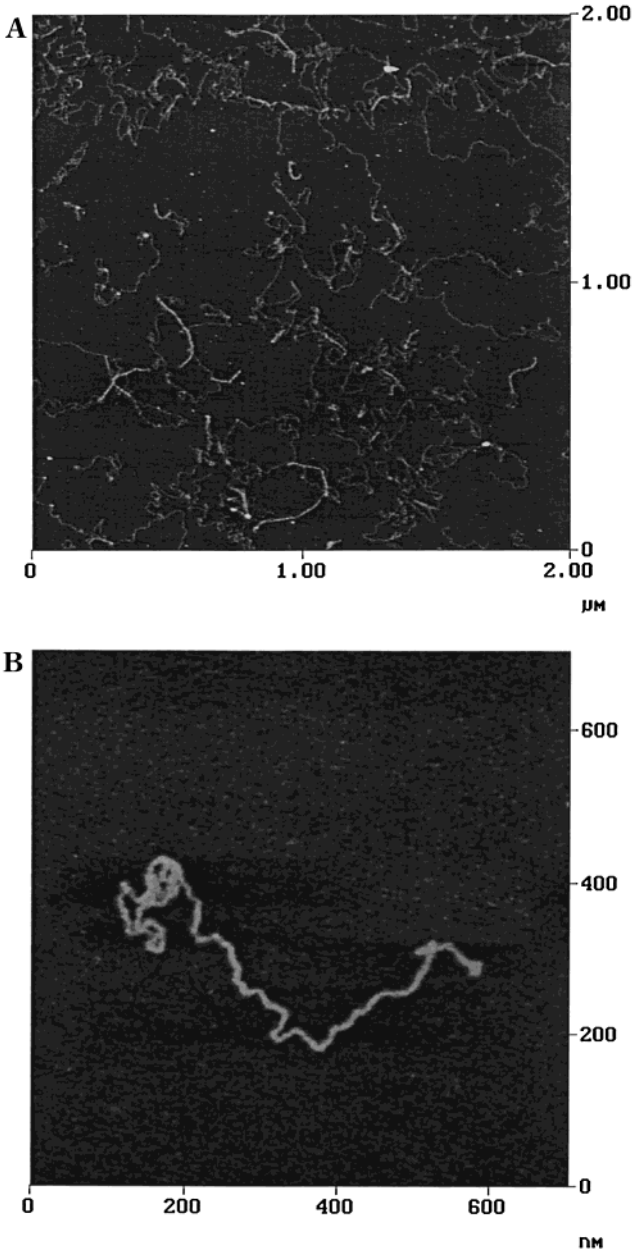

Figure 6. AFM images of succinoglycan (10 mg L^{-1}, dissolved in 0.01 M KCl) deposited on mica: (A) height mode image, scan size 2.0 μm × 2.0 μm; (B) height mode image, scan size is 705 nm × 705 nm.

disordered conformation than in its helical state. In the presence of salt, the increase in rigidity can be explained by the screening of the charges located on the side chains which allows the molecule to adopt an ordered conformation as a single helix at all polymer concentrations. In the absence of salt, a disordered, nonhelical conformation is favored due to the repulsion of the lateral chain segments. The characteristics of the different chains are summarized in Table 1.

The ratio of the even moments of the angular distribution $\langle\theta^4\rangle/\langle\theta^2\rangle^2$ was plotted as a function of contour distance (Figure 10) in order to test whether the distribution of angles along the chains was Gaussian. For the rigid chains observed in the absence of KCl, a value reasonably close to the theoretical value of 3 was found for contour distances below 150 nm. As mentioned earlier, this indicates that the polymer is at equilibrium with the mica surface which is likely due to the high

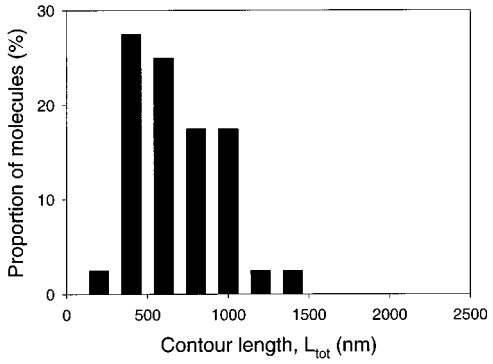

Figure 7. Histogram of the contour length of the "flexible" succinoglycan chains, dissolved in 0.01 M KCl and deposited on mica.

Table 2. Measured and Calculated End-to-End Distances of the Different Chains[a]

	calcd $\langle R_{ee}\rangle_{2D}$	calcd $\langle R_{ee}\rangle_{proj}$	measd $\langle R_{ee}\rangle$
rigid chains in water	487	281	496 ± 190
flexible chains in water	225	130	297 ± 120
flexible chains in 0.01 M KCl	265	153	178 ± 100

[a] All values in nm.

polymer rigidity under these conditions. On the other hand, in both water and 0.01 M KCl, the ratios of the even moments of the angular distributions for the flexible chains were significantly different from 3, indicating nonequilibrium deposition conditions.

As a further verification to determine whether the chains reached equilibrium on the mica, L_p and L_{tot} values were determined from the AFM images and employed to calculate the mean-square end-to-end distances according to the following equations:[31]

$$\langle R_{ee}^2\rangle_{2D} = 4L_pL_{tot}\left(1 - \frac{2L_p(1 - \exp(-L_{tot}/2L_p))}{L_{tot}}\right) \quad (2)$$

and

$$\langle R_{ee}^2\rangle_{proj} = {}^4/_3 L_pL_{tot}\left(1 - \frac{L_p(1 - \exp(-L_{tot}/L_p))}{L_{tot}}\right) \quad (3)$$

where L_{tot} is the contour length of the succinoglycan chains and $\langle R_{ee}^2\rangle_{2D}$ and $\langle R_{ee}^2\rangle_{proj}$ are the 2D and projected mean-square end-to-end distances. For polymer chains which have reached thermodynamic equilibrium on the surface, their conformation and thus their measured end-to-end distances are expected to correspond to the 2D conformation of the macromolecules rather than a 3D projection. For example, Rivetti et al.[31] have shown that DNA molecules adopted an equilibrated 2D conformation on mica, with measured L_p values from the AFM images in agreement with L_p values of DNA in solution and calculated $\langle R_{ee}\rangle_{2D}$ values equal to measured $\langle R_{ee}\rangle$ values. AFM derived experimental and calculated end-to-end distances for succinoglycan are given in Table 2. For the rigid chains in water, the measured $\langle R_{ee}\rangle$ distance is close to the calculated 2D $\langle R_{ee}\rangle$ distance, which indicates that the chains are likely equilibrated with the mica surface. For the flexible chains in water, the measured $\langle R_{ee}\rangle$ was slightly larger than the calculated values of $\langle R_{ee}\rangle_{2D}$. This finding combined with the results showing that the even moment of the angular distribution was different from

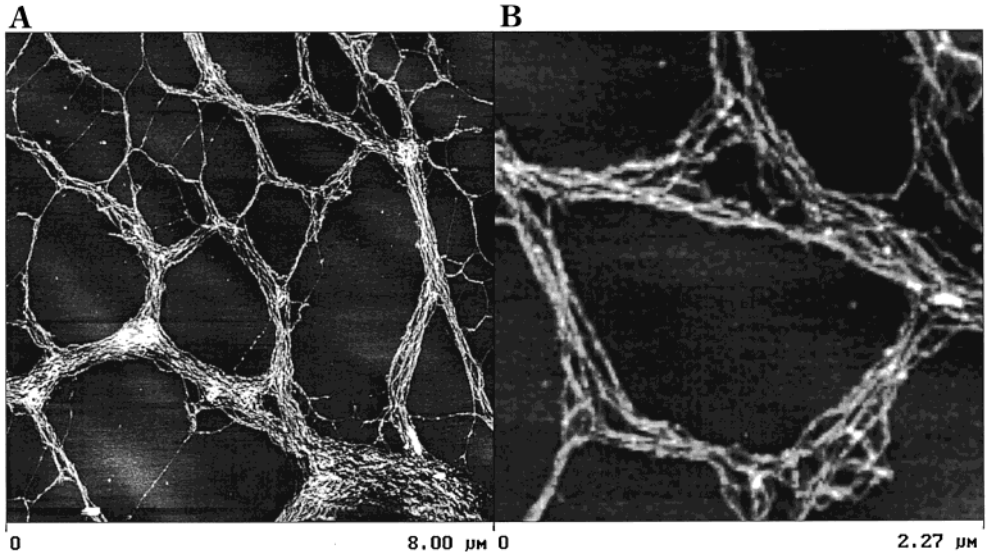

Figure 8. AFM images of succinoglycan (10 mg L^{-1}, dissolved in 0.5 M KCl) deposited on mica: (A) height mode image, scan size 8.0 μm × 8.0 μm; (B) height mode image, scan size 2.3 μm × 2.3 μm.

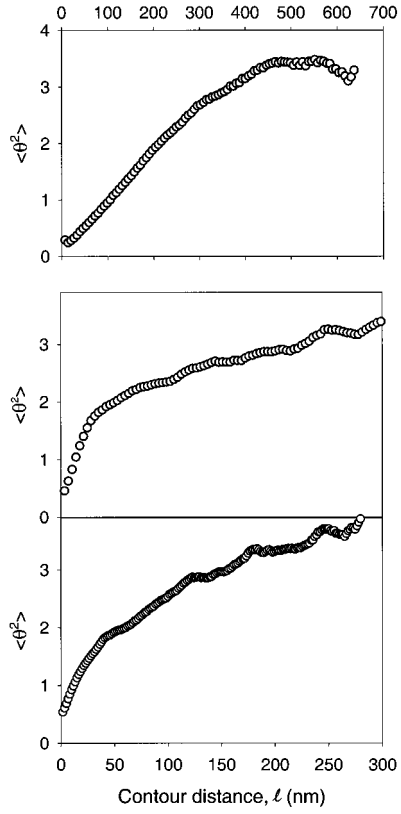

Figure 9. $\langle\theta^2\rangle$ versus the contour distance, *l*, for (A) "rigid" succinoglycan chain in water, (B) "flexible" succinoglycan chain in water, and (C) "flexible" succinoglycan chain in 0.01 M KCl.

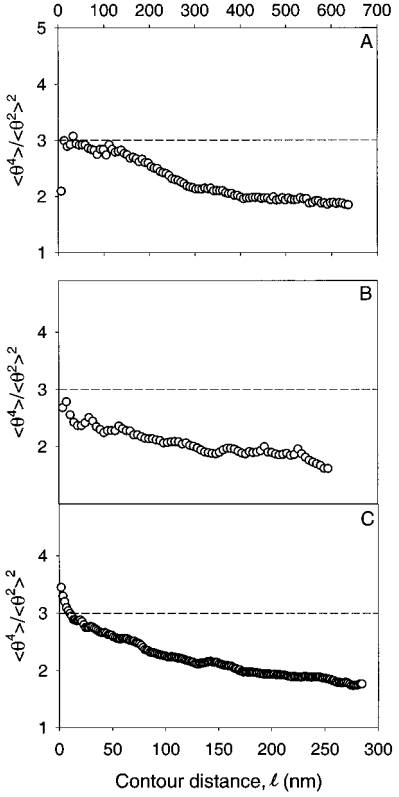

Figure 10. $\langle\theta^4\rangle/\langle\theta^2\rangle^2$ versus the contour distance, *l*, for (A) "rigid" succinoglycan chain in water, (B) "flexible" succinoglycan chain in water, and (C) "flexible" succinoglycan chain in 0.01 M KCl.

3 and with the observation of heterogeneous zones of polymer on the mica indicated that the succinoglycan was not at equilibrium with the mica surface. In this case, the flexible macromolecules are likely to adopt a more extended conformation than that occurring in solution due to a charge repulsion with the negatively charged mica. Another possibility that cannot be excluded is that the induced orientation could be partly an artifact of the drop deposition method which may give some orientation to poorly attached materials on the mica.

In 0.01 M KCl, the experimental value of the mean-square end-to-end distance for the chains was determined to be reasonably close to the projected 3D $\langle R_{ee}\rangle$ value. This indicates that the molecules may have been trapped on the mica prior to their equilibration possibly due to a reduction in the electrostatic repulsion between the polymer and the mica surface in the presence of salt.

Conclusions

The present study has demonstrated that AFM is a powerful technique for the analysis of the local confor-

mation of polymers at the nanometric scale. Several key results were demonstrated: (i) succinoglycan deposited from water was imaged both as individual single chains or as an association of at least two individual chains; (ii) increasing the ionic strength of the medium resulted in an increase of the intrinsic rigidity of the macromolecules, probably due to the formation of a single helix; (iii) AFM results were dependent upon both the substrate and the preparative technique which was employed. This third point merits particular attention. Although one of the main advantages of the AFM technique is its ability to provide information which cannot be obtained by averaging methods, for example, local information on the polymer structure and polydispersity, it was shown that in some cases equilibrium was not attained with the substrate, resulting in conformations that were not representative of those in solution. Furthermore, local increases in concentration due to the sample drying process are possible, again greatly complicating direct extrapolation of the results from bulk measurements made in dilute solution. This work thus emphasizes the complementarities of the AFM technique and conventional physical techniques for the determination of the size and shape of individual macromolecules and provided an example of the important role of substrates on the conformation of polymers at interfaces.

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