Visualization of Single and Aggregated Hulless Oat (*Avena nuda* L.) (1→3),(1→4)-*β*-D-Glucan Molecules by Atomic Force Microscopy and Confocal Scanning Laser Microscopy

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Surfactants were used to disperse oat β-glucan. Atomic force microscopy (AFM) images of the resulting samples revealed a distribution of extended chainlike molecules and allowed, for the first time, direct visualization of single oat β-glucan molecules with cross-sectional heights of about 0.44 nm. The number-average contour length (L_n) and root-mean-square end-to-end distance ($\langle R_{ee}^2 \rangle^{1/2}$) measured from the AFM images were 938 and 912 nm, respectively. The calculated persistence length (L_p) was 526 nm. The weight-average molecular weight (M_w) calculated from single β-glucan molecules was 4.43×10^5. Samples without surfactant showed a strong tendency to form aggregates. The sample concentration, reserving time, and calcofluor as well as freezing could affect the formation of aggregates. These aggregates were visualized by both AFM and confocal scanning laser microscopy. The shape of the aggregates changed from small dots with diameters of approximately 20−50 nm to microfibrils over 3 μm long with the increasing of the concentration of oat β-glucan from 10 to 100 μg/mL. The particle size distribution obtained by a laser particle size analyzer was 926 nm, which confirmed the size of oat β-glucan molecules obtained from AFM images.

INTRODUCTION

Hulless oat (*Avena nuda* L.) originated from China. It's an excellent source of dietary fiber components such as mixed link (1→3),(1→4)-*β*-D-glucan, which mainly exists in the subaleurone and endosperm cell wall of oat. Oat β-glucan has been shown to be an unbranched polysaccharide composed of D-glucopyranosyl residues arranged as mixtures of consecutively (1→4)-linked glucose units in blocks that are separated by single (1→3) linkages. The (1→4) linkages occur in groups of two or three (*1*), although much longer segments have been reported (*2*). Approximately 90% of the main chain consists of 3-O-*β*-cellobiosyl D-glucose and 3-O-*β*-cellotriosyl D-glucose, and their molar ratio was determined to be 2.0−2.4 (*1−3*). The presence of the (1→3) linkages in oat β-D-glucan is responsible for β-glucan's solubility in water as compared to related (1→4)-*β*-D-linked polymers such as cellulose and chitosan, which are also more ordered, fibrous, and water insoluble. The molecular weight of oat β-D-glucan reported in the literature distributed within the range of $0.065-3 \times 10^6$ (*4*).

Large amounts of research have been focused on oat β-glucan because the polysaccharide has lots of functions such as hypoglycemic activity, serum lipid-lowering capacity, and immune regulation effect. The bioactivities are related to the aqueous solution behavior of oat β-glucan. So understanding of both the single oat β-glucan molecule and the aggregates of the molecules in aqueous solution is the key to explain these functions of oat β-glucan.

Recent reports described the tendency of barley β-glucan molecules to aggregate together. By light scattering and viscometry on solutions of β-glucan extracted from beer, the solution behavior was explained by a modified fringed micelle structure for clusters (*5*). The viscoelastic behavior of three samples of oat β-glucan having different molecular weights indicated that only for low molecular weights did the system display gellike properties, owning to self-association through cellulose-like sequences (*6*). Rheological properties of barley β-glucan in aqueous solution have been studied. The results shed light on the association behavior of these macromolecules and even on the formation of network structures under certain circumstances (*7*).

Atomic force microscopy (AFM) has been applied successfully to visualize a range of polysaccharides including curdlan (*8*), gellan (*9*), xyloglucan (*10*), alginate (*11*), pectin (*12*), and

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schizophyllan (*13*). Potato starch was also observed by AFM (*14*). Three preparation methods (drop deposition, adsorption, and ultracentrifugation) were tested on two reference compounds, a humic substance and a polysaccharide, to obtain the optimized AFM images (*15*). Because the preparation methods were critical in avoiding artifacts of AFM images and obtaining the supposed form of macromolecules, the method should be carefully chosen in sample preparation. To produce single molecular aqueous solutions, surfactants were often added to the solution for dispersing macromolecules. Amylose has been visualized at the single molecular level by AFM. The method involved incubation of hot amylose solutions with iodine and the surfactant Tween-20, and no aggregation took place in this solution (*16*). More information could be obtained from AFM images, such as macromolecular chain contour length, end-to-end distance, and persistence length (L_p); the methods were described in the characterization of cartilage aggrecan macromolecules by Ng et al. (*17*) and a single molecule study of Xanthan conformation by Camesano et al. (*18*).

Calcofluor is a fluorescent probe capable of making hydrogen bonds with β-(1→4)- and β-(1→3)-linked polysaccharides. The fluorophore shows a high affinity for chitin, cellulose, and glucan, forming hydrogen bonds with free hydroxyl groups (*19*). Calcofluor was often used to analyze oat β-glucan (*20*). The fluorescence intensity of calcofluor increases when the fluorophore binds to oat β-glucan so that we can distinguish glucan from the background.

Confocal scanning laser microscopy (CSLM) is a useful method to visualize structures of a biosystem, and it can provide clear images and detailed morphology. CLSM has been used to study the behavior of mixtures of proteins, gelatine, whey proteins, and β-lactoglobulin and polysaccharide, dextran, gellan gum, carrageenan, gum Arabic, and starch (*21*).

MATERIALS AND METHODS

Isolation, Purification, and Characterization of Oat β-Glucan. β-Glucan was extracted from hulless oat (*A. nuda* L.) according to cryogelation of cereal β-glucans. Such behavior has been described by Morgan et al. (*22, 23*). The effects of molecular size and the fine structure of β-glucan on their cryogelation behavior were investigated by differential scanning calorimetry (*4*).

Hulless oat grain (Ziyuan 2001, obtained from Shanxi Plateau Plant Institute) was ground in a sample mill to pass a 0.45 mm screen. The flour (10 g) was extracted with distilled water (100 mL) at 50 °C for 1 h. The spent flour was centrifuged at 4000g for 20 min, and the supernatant was frozen at −18 °C for 20 h. The frozen solution was then thawed in a water bath at room temperature. The gelling material was recovered by filtration through a no. 3 sintered glass filter and washed with distilled water. The resultant product was purified by redissolving in 100 mL of water at 80 °C, centrifuging at 4000g for 10 min, and repeating the freeze−thaw steps two times. The final solution was passed through a 0.8 μm filter followed by condensation to about 10 mL, that is, 1/10 of the filtrate volume, and then freeze-dried.

The total sugars content in the oat β-glucan extract was 93.8% by means of phenol−sulfuric acid method. The content of reducing sugars and protein in the purified oat β-glucan was nearly 0 determined by Fehling's test and Komas blue reaction, respectively.

The purified oat β-glucan was analyzed via Waters HPLC system (Sugar Pak I column, 300 mm × 6.5 mm i.d., Waters). Double-distilled water was used as a mobile phase at a rate of 0.4 mL/min. The column temperature was 85 °C. A RI detector was applied to detect the monosaccharide. Glucose, mannose, galactose, and xylose were used as standards. Ten microliters of standard and 25 μL of sample were injected into the system. The resulting chromatogram indicated that oat β-glucan was a homosaccharide composed of glucose. The sample was mixed with KBr and pressed into a pellet and then scanned on a Nexus 670 FT IR spectrometer (Nicolet, Madison, WI). The spectra were recorded covering a range of 4000−400 cm^{-1}.

Oat β-glucan was dissolved in D$_2$O, transferred to a 5 mL sample tube, and then assayed on a Bruker DMX-500 NMR spectrometer; a ^{1}H NMR spectrum was obtained at 500 MHz, and a ^{13}C NMR spectrum was obtained at 125 MHz. IR and NMR spectra showed that oat β-glucan consisted of β-D-glucopyranose units joined by 1→4 and/or 1→3 linkages. The ratio of β-(1→4) linkages to β-(1→3) linkages was approximately 2.1:1 obtained from the ratio of intensity of the two types of linkages in ^{13}C NMR spectrum.

The molecular weight of oat β-glucan was determined on GPC-LLS (DLS-700, Otsuka Electronics, Japan). The system included a DAWN-DSP multiangle light scattering detector, P100 pump, TSK-GEL G6000PWXL column (7.8 mm × 300 mm), and RI detector. The sample was analyzed at 25 °C using 0.2 mol/L NaCl as the mobile phase at a flow rate of 1.0 mL/min. The laser light wavelength was 632.8 nm. Astra software was applied to process the data obtained. The resulting weight average molecular weight was 4.49×10^5, with a polydispersity index of 1.32.

Sample Preparation for AFM Imaging. Oat β-glucan was dissolved in distilled water to make a 1 mg/mL solution. After it was heated to 80 °C for 1 h with constant stirring, the solution was diluted stepwise to 100, 50, 20, 10, and 1 μg/mL. To deposit oat β-glucan onto newly cleaved sheets of mica, 1 μL of oat β-glucan solution was pipetted briefly onto the mica surface in order to keep the solution hot. The surface was air-dried for 2 h in a dust-free enclosure and then imaged using AFM. Aliquots of prepared oat β-glucan solutions were stored at room temperature for 9 days and imaged by AFM.

However, the above method could only provide aggregates of oat β-glucan molecules. To obtain single molecules, surfactants such as SDS, Tween-20, and Tween-80 were added to the 1 mg/mL glucan solution at a ratio of 1:1 (surfactants:glucan/w:w) and heated together. Then, 80 °C distilled water was used to dilute the solution to a final concentration of 1 μg/mL. Finally, about 1 μL of the diluted oat β-glucan solution was pipetted onto the newly cleaved mica and air-dried. The process was performed briefly to avoid cooling of the solution and the formation of aggregates of oat β-glucan so as to obtain single glucan molecules.

Calcofluor (Sigma, F3543, St. Louis, Missouri) was dissolved in pH 10 Na$_2$CO$_3$−NaHCO$_3$ (0.1 mol/L) aqueous solution and formed a transparent fluorescent probe solution. A 20 μg/mL calcofluor solution was mixed with the same volume of 20 μg/mL oat β-glucan solution without surfactant to make a final concentration of 10 μg/mL combined β-glucan-calcofluor solution. One microliter of the combined solution was pipetted onto the mica surface immediately after combination. The mica surface was rinsed with distilled water for 5 s and then air-dried and imaged by AFM.

AFM Imaging Procedures. Samples were examined using an AJ-III Atomic Force Microscope (AJ Nano-Science Development Co. Ltd., Shanghai, China) in tapping mode. About 1 μL of diluted solutions was immediately spread onto freshly cleaved mica surfaces and imaged by AFM. AFM imaging was made at room temperature in atmosphere. Samples were placed on top of the piezoelectric scanner and scanned using a Si cantilever with a quoted spring constant of 23−91 N/m at a cantilever driving frequency of 315 kHz and a scanning frequency of 1−2 Hz. AJ online software and AJ offline software (AJ Nano-Science Development Co. Ltd.) were used to obtain and process AFM images.

Single Chain Statistics from AFM Images. The method to obtain the contour length and end-to-end distance was described by Ng (*17*) and Camesano et al. (*18*). SigmaScan Pro 5.0 image analysis software (SPSS Science, Chicago, IL) was used to analyze the AFM images. The traces of single oat β-glucan molecules were digitized into a line with one pixel width. The contour lengths, L_c, and end-to-end distances, R_{ee}, were measured directly from these images.

The degree of polydispersity in the polymer sample was defined as the ratio of the weight-average contour length (L_w) and number-average contour length (L_n), as

$$\text{polydispersity index} = \frac{L_w}{L_n} = \frac{M_w}{M_n} \qquad (1)$$

where $L_n = \sum_n n_i L_i / \sum_n n_i$ and $L_w = \sum_n n_i L_i^2 / \sum_n n_i L_i$.

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The expected average length and molar mass of a repeating unit in oat β-glucan molecules were 1.9 nm and 592 g/mol, respectively (*5*). On the basis of these values, the weight-average molecular weight (M_w) and number-average molecular weight (M_n) can be estimated as $M_w = (592/1.9)L_w$ and $M_n = (592/1.9)L_n$.

The L_p calculation was based on the following assumptions (*18*): (i) The probability density $P[\theta(l)]$ of the bend angle $\theta(l)$ between consecutive links of the chain is Gaussian (eq 2), as shown below:

$$P[\theta(l)] = \left(\frac{L_p}{2\pi l}\right)^{1/2} \exp\left(-\frac{L_p\theta^2}{2l}\right) \tag{2}$$

where θ is the bend angle, l is the distance between segments, and L_p is the persistence length of the polymer chain. (ii) The observed two-dimensional (2D) conformation is obtained by permitted deformations and is not a projection of the three-dimensional (3D) structure. (iii) The adsorbing surface does not alter the local rigidity of the polymer chain.

The definition of eq 3 was used to calculate L_p, while eq 4 was applied to check if the first assumption was valid.

$$\langle\theta^2(l)\rangle = \frac{l}{L_p} \tag{3}$$

$$\langle\theta^4(l)\rangle/\langle\theta^2(l)\rangle^2 = 3 \tag{4}$$

To test assumptions ii and iii, a calculation was made to determine whether the polymer chain reached an equilibrium conformation on the surface of mica. The equilibrium conformation of polymer chains on the surface will correspond to a 2D conformation rather than a 3D projection. The end-to-end distances corresponding to 2D and projected conformations are presented below:

$$\langle R_{ee}\rangle_{2D}^2 = 4L_p L\left[1 - \frac{2L_p}{L}(1 - e^{-L/2L_p})\right] \tag{5}$$

$$\langle R_{ee}\rangle_{proj}^2 = \frac{4}{3}L_p L\left[1 - \frac{L_p}{L}(1 - e^{-L/L_p})\right] \tag{6}$$

Sample Preparation for CSLM Imaging. The effects of concentration, reserving time, and freezing on the aggregation state of oat β-glucan molecules were visualized by CSLM. The final concentrations of oat β-glucan were 5, 10, 15, 40, 60, 80, and 100 μg/mL, respectively. The oat β-glucan solutions were mixed with calcofluor in pH 10 Na$_2$CO$_3$–NaHCO$_3$ solution at a ratio of 1:1 (V:V), and then, the blended solutions were pipetted onto microscope glass slides and imaged with CSLM. A 80 μg/mL oat β-glucan solution was stored at room temperature for as long as 35 days and imaged at 0, 2, 5, 25, and 35 days to see the aggregates of oat β-glucan. To test the effect of freezing on oat β-glucan solutions, 80 μg/mL oat β-glucan solutions were frozen in a refrigerator at −18 °C for 20 h, and the frozen solution was thawed at room temperature and mixed with calcofluor solution described as above and then imaged with CSLM.

CSLM Imaging Procedures. Samples were imaged by a confocal scanning laser microscope (Leica SPZ, Germany). The sample solution was pipetted into the well of the microscope glass slide, and then, a cover glass was carefully put onto the well and imaged using CSLM. The excitation wavelength was 340 nm, and the emission wavelength was 440 nm.

Measurement of Particle Size Distribution of Oat β-Glucan in Aqueous Solution. Oat β-glucan (10 mg) was dissolved in 5 mL of distilled water and kept at 80 °C for 3 h in a water bath. After it cooled to room temperature, the solution was diluted to 1, 10, and 100 μg/mL and then tested for particle size distribution using laser particle size analyzer (Beckman Coulter N4 plus submicron particle sizer, Beckman Coulter, United States) at 25 °C with the laser wavelength at 632.8 nm and a scattering angle of 90°; each solution was tested three times to ensure that it reached equilibrium.

RESULTS

Aggregations of Oat β-Glucan Molecules at Various Concentrations. Oat β-glucan molecules dispersed in hot water

tended to form clusters even in dilute solutions. An AFM image of 10 μg/mL oat β-glucan is shown in **Figure 1A**; there were many small dotlike aggregates with diameters of about 20−50 nm and cross-sectional heights of about 3−5 nm. Those small spherical aggregates may be entangled chains of oat β-glucan molecules in aqueous solution. However, the spherical aggregates collapsed to a flat shape when drying in air. Another important reason to form the flat shape may be the width amplification effect of the tip of the AFM cantilever. With an increase in the concentration of oat β-glucan solutions, larger aggregates began to form just like the AFM images shown in **Figure 1B** (20 μg/mL oat β-glucan) and **C** (50 μg/mL oat β-glucan). The diameters of the aggregates in **Figure 1B** distributed from 200 to 800 nm while the aggregates in **Figure 1C** had relatively uniform diameters from 600 to 800 nm. The cross-sectional heights of the aggregates in **Figure 1C** distributed quite uniformly around 3.6 nm. Thick long microfibrils over 3 μm long were clearly shown in **Figure 1D** when the oat β-glucan concentration reached 100 μg/mL; the microfibrous structures were about 20 nm high and 300 nm wide. From the changes of the diameters and heights, it could be deduced that more condensed and compact aggregates formed with the increasing concentration of oat β-glucan. The aggregation effect attributed to the intense intermolecular hydrogen bond interactions of oat β-glucan. With the rise of the concentration, glucan molecules had more chances of thermal motion and collision, which led to formation of larger aggregates. These AFM images give direct evidence of the trend to form aggregates for oat β-glucan.

Aggregations of Oat β-Glucan Molecules at Various Times. A number of platelike structures with diameters of about 300 nm and cross-sectional heights of 15−20 nm are presented in the AFM image (**Figure 2**) of 10 μg/mL oat β-glucan aqueous solution, which were stored at room temperature for 9 days. It indicated that aggregation was a spontaneous course and glucan molecules gradually collided into each other and associated with each other to form relatively stable structures by hydrogen bonds, although it was a slow process. The AFM images agree with the results of Morgan and Robert (*23*).

Aggregations of Oat β-Glucan Molecules Combined with Calcofluor. A 10 μg/mL concentration of calcofluor in pH 10 Na$_2$CO$_3$–NaHCO$_3$ aqueous solution pipetted onto the mica surface looked like granules as shown in **Figure 3A**. These granules were about 6−20 nm in cross-sectional height and 120 nm in diameter. An AFM image of 10 μg/mL calcofluor−glucan complex is shown in **Figure 3B**. It seemed that the granules joined together to form wormlike chains with the cross-sectional height of 50−100 nm. The strange structures were ascribed to the combination of calcofluor molecules on the chains of oat β-glucan molecules. Calcofluor molecules also could connect several oat β-glucan molecules together to form large aggregates, which were indicated through the change of the heights of the objects in AFM images.

Single Oat β-Glucan Molecules Dispersed with Surfactants. Sodium dodecyl sulfate (SDS), a common surfactant, was used to disperse oat β-glucan aggregates. **Figure 4A−D** shows a series of representative AFM images obtained after treatment of oat β-glucan solutions with SDS. Only discrete and extended polymer chains were present in the AFM images. The chains were approximately 0.44 ± 0.1 nm in height and 2.8 nm in width according to height and width analysis. The result is consistent with a similar glucopyranosyl molecule, scleroglucan, which is a (1→6)-branched (1→3)-β-D-linked glucan, having a single strand polymer height of 0.55 nm based on X-ray

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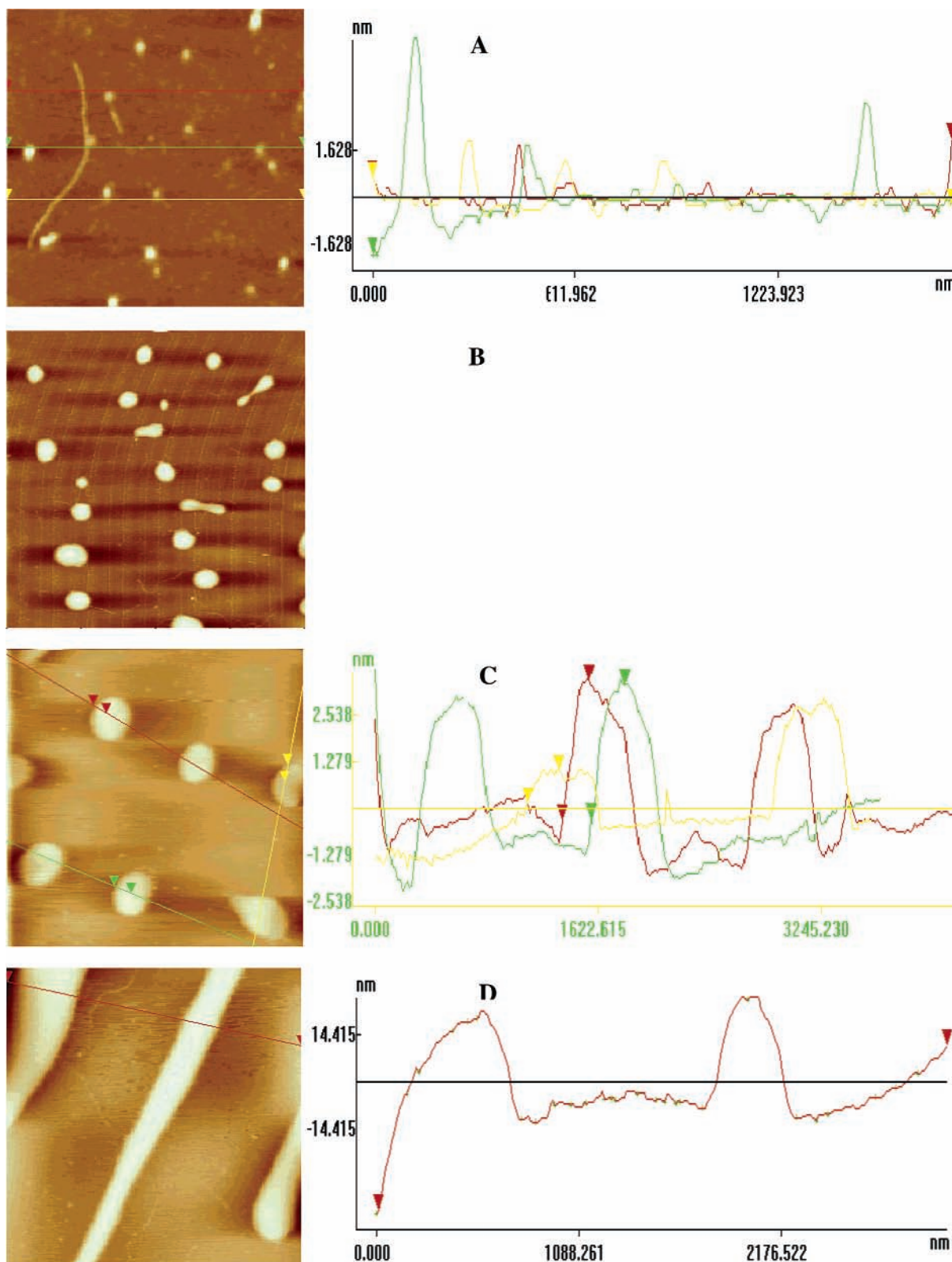
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Figure 1. AFM images of oat β-glucan dispersed in 80 °C distilled water with the final concentration of (**A**) 10 μg/mL, scan size: 1.8 μm × 1.8 μm; (**B**) 20 μg/mL, scan size: 6.3 μm × 6.3 μm; (**C**) 50 μg/mL, scan size: 4.0 μm × 4.0 μm; and (**D**) 100 μg/mL, scan size: 3.0 μm × 3.0 μm. The cross-sectional profile is shown on the right of each AFM image, and the curves with different colors in the cross-sectional profile correspond to the line of the same color in the AFM images on the left.

diffraction measurements (*24*). The height of the oat β-glucan molecule is lower than that of scleroglucan because the former is a single strand polymer without a branch while the latter is a similar single strand polymer with branches. In a word, it can be deduced from the height data that oat β-glucan is a single strand polymer. The result verified the structure of oat β-glucan molecule presented in many literatures.

Because oat β-glucan molecules appeared as discrete and extended entities in AFM images, it was possible to determine the distribution of the contour lengths and end-to-end distances
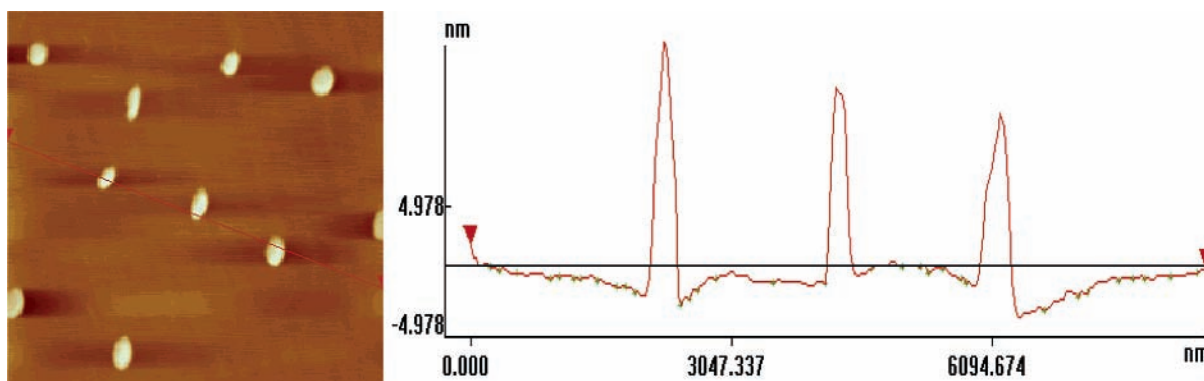
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Figure 2. AFM image and cross-sectional profile of 10 μg/mL oat β-glucan stored at room temperature for 9 days; scan size, 8.0 μm $\times$ 8.0 μm.

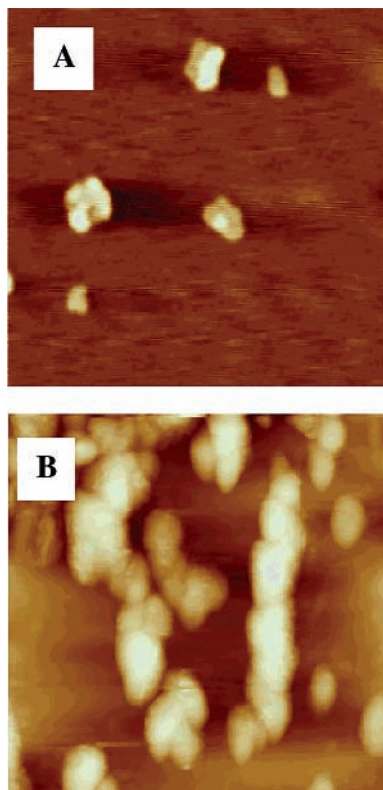**Figure 3.** AFM images of (**A**) 10 μg/mL calcofluor in pH 10 Na_2CO_3–$NaHCO_3$, scan size: 1.5 μm $\times$ 1.5 μm; (**B**) 10 μg/mL calcofluor in pH 10 Na_2CO_3–$NaHCO_3$ combined with 10 μg/mL oat β-glucan, scan size: 1.5 μm $\times$ 1.5 μm.

of the polymer. Through analysis of a number of single molecules, the contour lengths, L_c, were measured directly from these images and the probability distribution histogram was calculated. The contour lengths distribution is shown in **Figure 5**; $L_n = 938$ nm, and the number of the chains is 69. The end-to-end distances, R_{ee}, were measured for the same population of chains and are shown in **Figure 6**; $\langle R_{ee}^2 \rangle^{1/2} = 912$ nm. According to IUPAC definition, contour length means the maximum end-to-end distance of a linear polymer chain, and root-mean-square end-to-end distance means the square root of the mean-square end-to-end distance of a linear polymer chain averaged over all conformations of the chain. So the oat β-glucan molecule is quite a rigid linear chain since the $\langle R_{ee}^2 \rangle^{1/2}$ is 97.2% of the L_n. It seems that both the contour lengths and the end-to-end distances are not quite uniform and have wide polydispersity probably due to the inherent property of the chains and degradation of oat β-glucan by endogenetic enzyme in the course of extraction. From the histogram of contour lengths

shown in **Figure 5**, it is possible to calculate the M_w and M_n. The expected average length and molar mass of a repeating unit were 1.9 nm and 592 g/mol, respectively (*5*). L_w was calculated to be 1422 nm. On the basis of these values, the calculated M_w was 4.43×10^5, which was in good agreement with the value 4.49×10^5, obtained from light scattering data of the same oat β-glucan sample. The calculated polydispersity index M_w/M_n was 1.52, which was similar to the polydispersity index (1.32) obtained from light scattering data.

L_p was calculated according to eq 3, and the inverse slope of a plot of $\langle \theta^2 \rangle$ vs l is equal to L_p. Values for l were chosen from $\sim$5 to 40% of the total contour length, and L_p values were calculated to be 526 nm from **Figure 7**. Although the intercept of the fitting curve is not zero, it can be explained that this deviation is the result of experimental error originating from the resolution limitations.

To check if the distribution of the bend angles was Gaussian, eq 4 was used. Values of $\langle \theta^4 \rangle / \langle \theta^2 \rangle^2$ should be equal to 3. While our values approached 3, some offset was seen (**Figure 8**). The trendline seemed to fit a natural logarithm equation shown in **Figure 8**. Camesano et al. (*18*) also observed similar offsets from Gaussian behavior in their analysis of xanthan conformation.

Calculated root-mean-square end-to-end distances corresponding to 2D- and 3D-projected conformations were 803 and 583 nm; it's obvious that the measured root-mean-square end-to-end distance (912 nm) was closer to the value calculated based on a 2D conformation on the surface. So the measured end-to-end distance was an equilibrium measurement.

Tween 20 and Tween 80 were also used to disperse oat β-glucan molecules, but the chains were over 1.0 nm in height, indicating that these chains were not single molecules but probably associations or helices composed of several molecules. The oat β-glucan aqueous solution became turbid when Tween 20 was mixed, but the solution kept clear when SDS was mixed, showing that Tween 20 and Tween 80 were not as effective as SDS to disperse oat β-glucan into single molecules. The discrepancy between the ability of the three surfactants to disperse oat β-glucan molecules may be related to their hydrophobic effects and charge in the surfactant molecules. Tween 20 and Tween 80 are neutral molecules, and they are more hydrophobic than SDS, while SDS is an ionic surfactant and it is smaller in size. So SDS can enter the space between oat β-glucan molecules more easily and break the hydrogen bonds by binding to oat β-glucan molecules and then forming charge repulsion between the chains of oat β-glucan molecules.

Interestingly, fringed micelle structures (*5*) were observed in 5 μg/mL oat β-glucan dispersed with 5 μg/mL SDS. The structure is shown in **Figure 9A**, where side-to-side aggregation

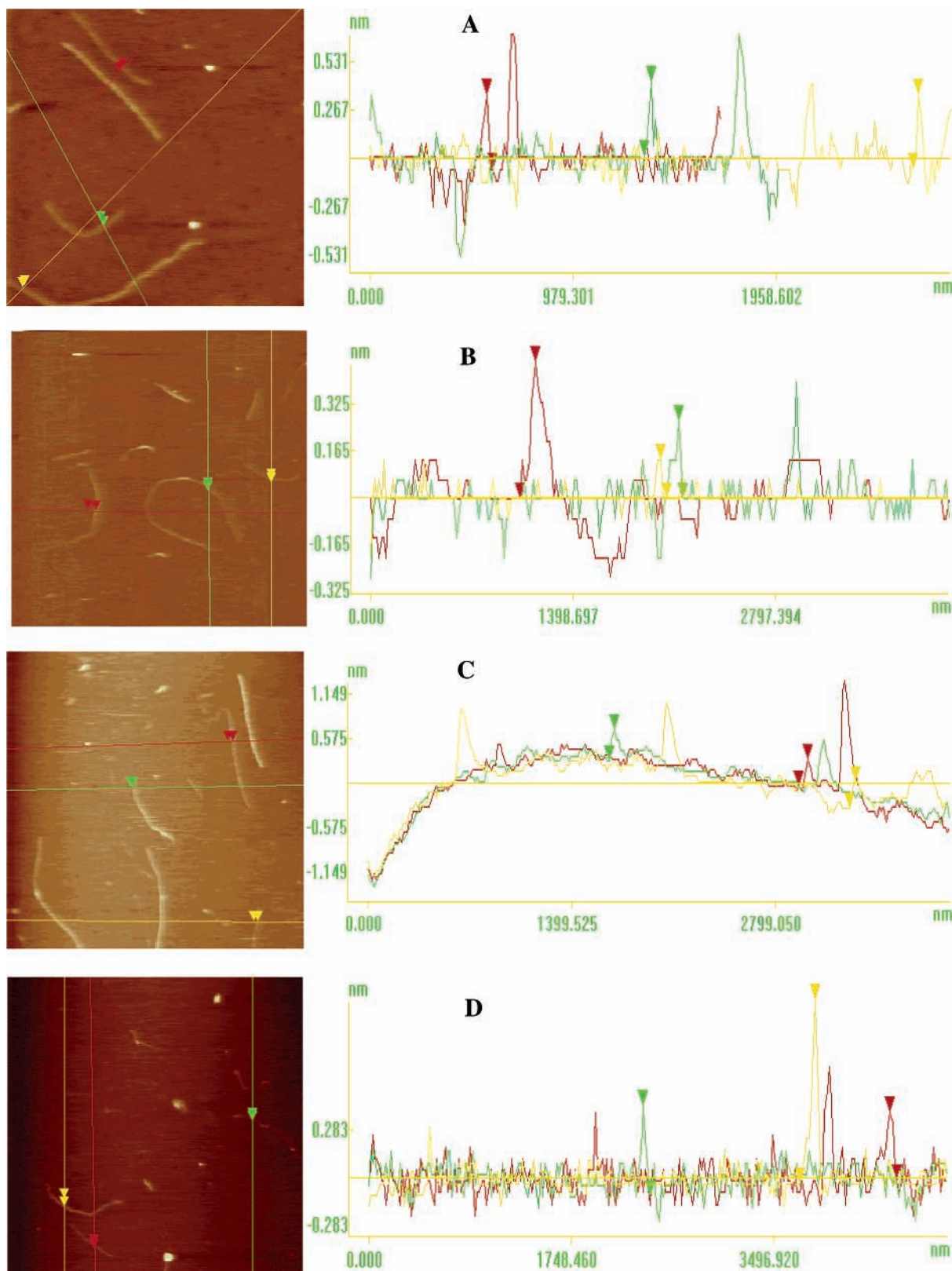**Figure 4.** AFM images of 1 μg/mL oat β-gluan dispersed with 1 μg/mL SDS at 80 °C for 1 h. (**A**) Scan size, 2.0 μm × 2.0 μm; (**B**) scan size, 4.0 μm × 4.0 μm; (**C**) scan size, 4.0 μm × 4.0 μm; and (**D**) scan size, 5.0 μm × 5.0 μm.

of chains occurred. In the outer region of the micelle, the chain sections kept their flexibility while within the "stem" the chain sections became stiffened. **Figure 9B** shows a schematic representation of such fringed micelles at two different aggregation stages. Clearly, there will be only a weak increase in the dimensions when more and more chains are laterally aligned, once a fairly rigid stem has been formed. A modification of the model of the fringed micelle is also depicted on the rightmost of **Figure 9B**, which differs from those on the left by a growth in thickness and length as molar mass is increased.

CSLM Images of Oat β-Glucan at Various Concentrations. Although calcofluor was widely used to dye cell wall polysaccharides in histology and to measure the content of β-glucan in cereal, it was rarely applied to visualize the
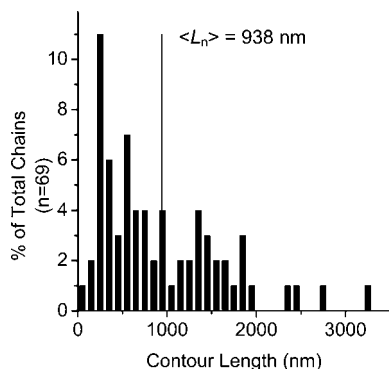
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Figure 5. Distribution of contour lengths for oat β-glucan chains. $L_n =$ 938 nm, and the number of the chains was $n = 69$.
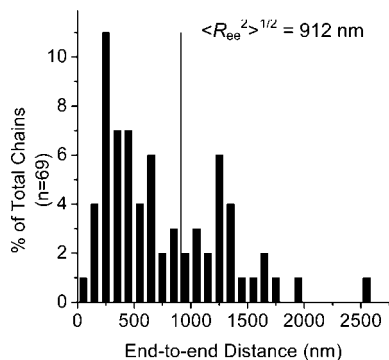

Figure 6. Distribution of end-to-end distances for oat β-glucan chains. $\langle R_{ee}^2 \rangle^{1/2} = 912$ nm, and the number of the chains was $n = 69$.
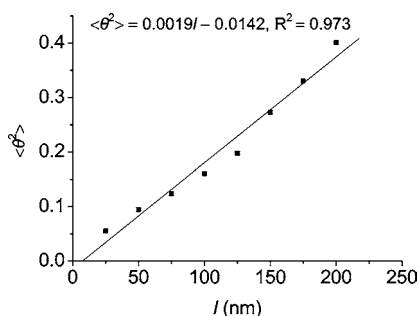

Figure 7. $\langle \theta^2 \rangle$ vs the contour distance, l; the linear regression equation is shown above the fitting curve.
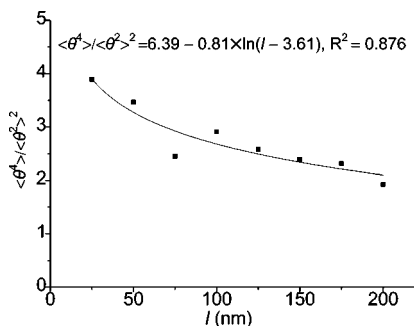

Figure 8. $\langle \theta^4 \rangle / \langle \theta^2 \rangle^2$ vs the contour distance, l; the natural logarithm regression equation is shown above the fitting curve.

aggregates of oat β-glucan in aqueous solution. So it is an attempt to visualize oat β-glucan by calcofluor in the experiment. Oat β-glucan at the concentration from 5 to 100 μg/mL was observed using CSLM. The images are shown in **Figure 10A–G**, and they clearly demonstrate that the amount of fluorescent dots of oat β-glucan combined with calcofluor increased with a rise in the concentration of oat β-glucan. So the intensity

increment of the fluorescence was in direct proportion with the concentration of oat β-glucan. That is the base of the fluorescent method for quantifying oat β-glucan in aqueous solution.

CSLM Images of Oat β-Glucan at Various Times. Oat β-glucan aqueous solution with the concentration of 80 μg/mL was stored at room temperature and imaged by CSLM at 0, 2, 5, 25, and 35 days. Aggregates of oat β-glucan looked like small dots at 0 and 2 day as in **Figure 11A,B**. The microfibrous structures began to form since day 5 as shown in **Figure 11C**. Then, these structures grew into thick fibers and tangled together to form gel at day 35 (**Figure 11E**). The above results were in accordance with the trend of oat β-glucan to form fibers in aqueous solution. These CSLM images showed the aggregates of oat β-gluan at a relative large scale and could be a useful complement for AFM images.

CSLM Images of Oat β-Glucan after Frozen for 20 h and Thawed. Once oat β-glucan aqueous solution was frozen and thawed, microfibrous structures would be formed in the solution. The CSLM images clearly showed the cryogelation of oat β-glucan at the concentration of 80 μg/mL (**Figure 12**). There seems to be two stages (25) to this process: (i) partial formation of a weak gel occurs rapidly during freezing and (ii) a slower process of additional growth of cryostructures takes place during thawing. The cryostructures of oat β-glucan were obvious fibers against the background of fluorescent dots, which meant not all of the oat β-glucan could form cryostructures. The cryogelation of oat β-glucan is related to molecular weight, concentration, and freezing and thawing conditions (4).

Particle Size Distribution of Oat β-Glucan in Aqueous Solution. It is clearly shown in **Figure 13** that the particle size of oat β-glucan in aqueous solution increased slightly with a rise in the concentration from 1 to 10 μg/mL. However, the particle size increased significantly when the concentration rose to 100 μg/mL in aqueous solution. This suggested that the degree of aggregation correlated with the concentration of oat β-glucan aqueous solution. It was noted that the particle size of oat β-glucan in aqueous solution (1 μg/mL) was 926 $\pm$ 28 nm, which was in accordance with the L_n (938 nm, measured from AFM images) of single molecular chain of oat β-glucan. The particle size measured from AFM images (20–50 nm) was much smaller as compared to the particle size obtained in aqueous solution (1191 $\pm$ 78 nm) when the concentration of oat β-glucan was 10 μg/mL. The difference indicated that oat β-glucan molecules changed from loose linear structures to compact spherical structures when oat β-glucan solution (10 μg/mL) was pipetted onto the mica surface and air-dried. Evaporation of water from thin layer of oat β-glucan solution may cause condensation and intramolecular hydrogen bonds interaction and may form flat structures 20–50 nm in diameter and 3–5 nm in height. Another reason involved may be the effect of surface tension in the course of water evaporation. When the concentration increased to 100 μg/mL, the particle size rose to 2023 $\pm$ 180 nm in aqueous solution while the lengths of microfibrous structures were over 3000 nm in AFM images. This meant that oat β-glucan aggregates associated together to form gellike fibers.

DISCUSSION

Concentration, Reserving Time, and Freezing-Induced Aggregation of Oat β-Glucan Molecules. Particle sizes of oat β-glucan increased with a rise in the concentration of the polymer aqueous solution from both particle size analyzer data and AFM images. However, the results did not seem to be consistent with each other when the concentration was 10 μg/ mL. Particle sizes obtained from particle size analyzer and AFM
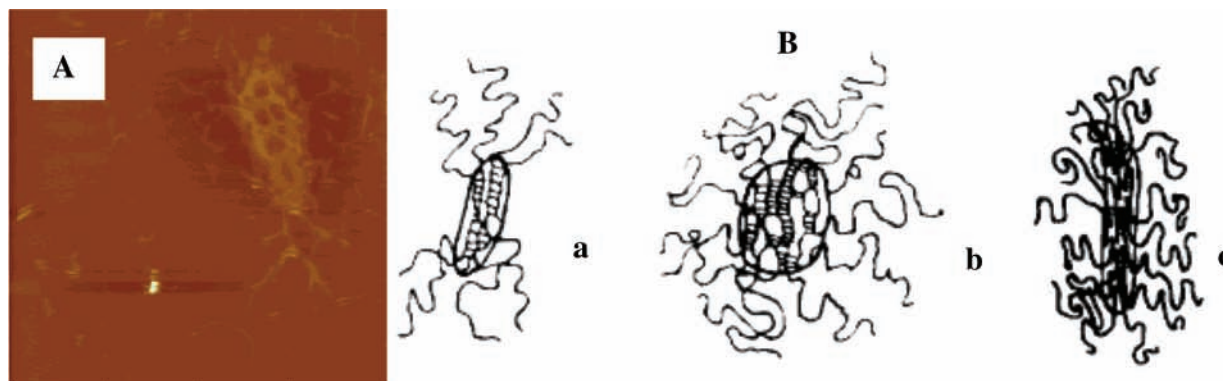
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Figure 9. (**A**) Fringed micelle structure observed in 5 μg/mL oat β-glucan dispersed with 5 μg/mL SDS; scan size, 8.0 μm × 8.0 μm. (**B**) Fringed micelles at two different aggregation stages (a,b) and a modified fringed micelle (c). The model of fringed micelle structures was copied from Grimm, A.; Kruger, E; Buchard, W. *Carbohydr. Polym.* **1995**, *27*, 205–214.
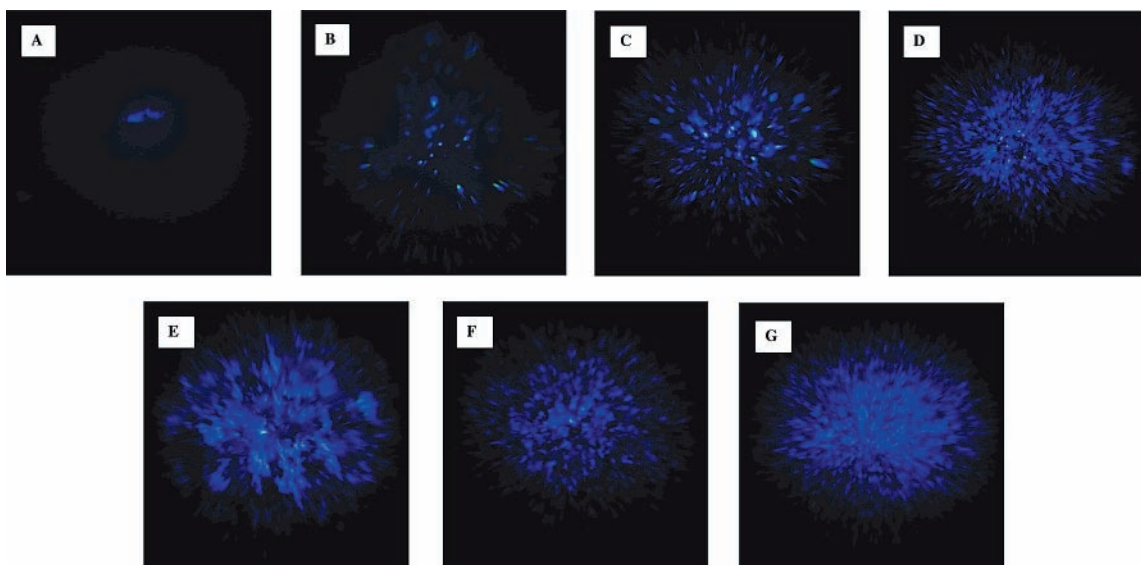

Figure 10. CSLM images of oat β-glucan at the concentration of (**A**) 5, (**B**) 10, (**C**) 15, (**D**) 40, (**E**) 60, (**F**) 80, and (**G**) 100 μg/mL. The size of all of the images was 300 μm × 300 μm.

images were 1191 and 20−50 nm, respectively. In addition to reflecting different molecular conformations between in aqueous solution and under AFM conditions, it gave us implications that drop deposition was not an ideal method in AFM sample preparation because glucan molecules probably changed their conformations in the course of evaporation of water from mica surface. The adsorption method can at least partly avoid conformational change and be a better choice in AFM sample preparation. Because of resolution limitations, CSLM could only illustrate oat β-glucan aggregates above the micrometer scale but not detailed conformational changes.

As the time passed by, glucan molecules tended to aggregate spontaneously indicated by both AFM and CSLM. Fibrous structures as long as several hundred micrometers formed after 5 days (**Figure 11C**) and gellike structures appeared 35 days later (**Figure 11E**) in 80 μg/mL glucan solution. These structures could not be included in AFM images for scanning scope limitations. After the glucan solution was frozen for 20 h and thawed, microfibrils began to form (**Figure 12**) while only small dots appeared in the glucan solution without freezing (**Figure 11A,B**).

Single Molecular Analysis. Cross-sectional heights were used to evaluate the thicknesses of the polymer chains because the AFM that we used had a higher resolving power (0.1 nm) in the vertical direction than in the horizontal direction (0.2 nm);

moreover, vertical heights could avoid the width amplification effect of the tip of the cantilever. The mean cross-sectional height was 0.44 nm, implying that the observed oat β-glucan chain was single strand as compared to scleroglucan, which was 0.55 nm in height (*24*).

The M_w (4.43 × 10^5) and polydispersity index (1.52) calculated based on L_w (1422 nm) were consistent with the results from light scattering (M_w = 4.49 × 10^5 and polydispersity index = 1.32). The similar results certified that oat β-glucan was a single strand molecule. If a single strand molecular chain formed a helix with itself by bending, the contour length would be shorter than that of the single chain and the calculated M_w would be lower than the M_w measured by light scattering; another probability is that if more than one single molecule associated to form a helix, the contour length of the helix would be longer than that of the single chain and the calculated M_w would be higher than the measured M_w obtained from light scattering; the third probability is that if several molecular chains of the same lengths formed a helix, the contour length of the helix would be the same as that of the single molecular chain, but the probability would be rather low.

$\langle R_{ee}^2 \rangle^{1/2}$ was 912 nm, reaching 97.2% of the L_n. The result suggested that the polymer chain existed as a linear conformation in aqueous solution. It was reasonable to deduce that SDS bound to oat β-glucan molecules and gave rise to intermolecular and
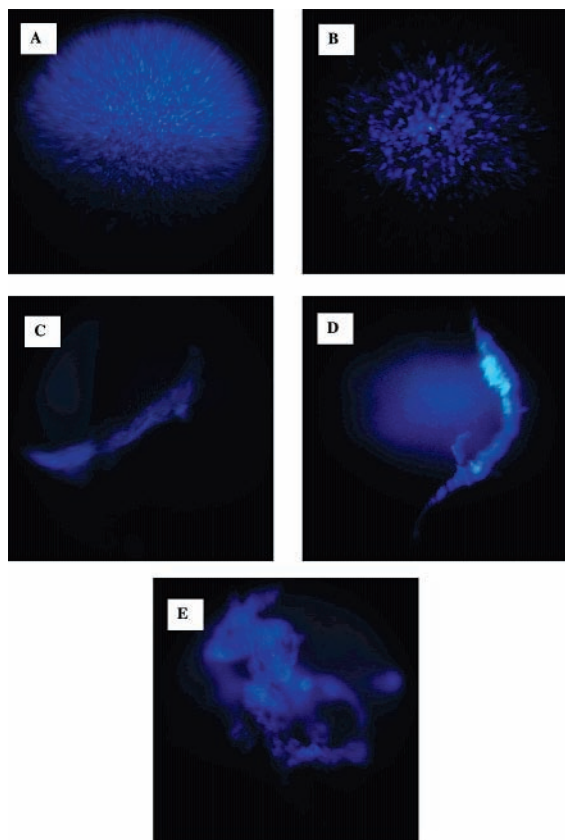
Visualization of Single and Aggregated Hulless Oat

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Figure 11. CSLM images of oat β-glucan with the concentration of 80 μg/mL stored at room temperature for (**A**) 0, (**B**) 2, (**C**) 5, (**D**) 25, and (**E**) 35 days. The size of all of the images was 300 μm × 300 μm.
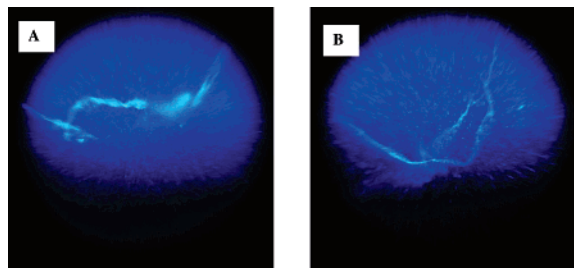
Figure 12. CSLM images of 80 μg/mL oat β-glucan after frozen for 20 h and thawed. Parts **A** and **B** were two typical microfibrous structures. The size of all of the images was 300 μm × 300 μm.
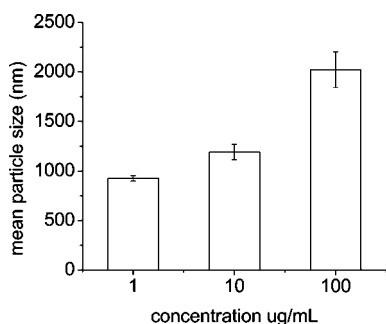

Figure 13. Particle size distribution of oat β-glucan in aqueous solutions with the concentration of 1, 10, and 100 μg/mL.

intramolecular charge repulsion, so the glucan molecules seemed to stretch to nearly full contour length and keep a stiff conformation.

The L_p of oat β-glucan was 526 nm, making the molecule even stiffer than xanthan, scleroglucan, and schizophyllan; the

L_p is 417 nm for xanthan in pure water (*18*) and approximately 200 nm for scleroglucan and schizophyllan (*26*). So oat β-glucan was quite a rigid polymer. The single molecular parameters suggested that the oat β-glucan molecule was a rigid rodlike single chain.

Oat β-glucan aggregates still could not be thoroughly broken into monomers even when SDS was mixed into aqueous solution and stirred for 1 h at 80 °C. The representative aggregation structure is shown in **Figure 9A**; the aggregate was similar with the fringed micelle structure presented by Grimm et al. (*5*) from light scattering and rheological methods. It's important to dilute glucan solution with hot water and pipet about 1 μL of the solution onto the mica surface immediately to avoid cooling of the solution since insoluble fibers were seen at the bottom of the glucan solution after sitting in an ambient environment for a night.

ABBREVIATIONS USED

AFM, atomic force microscopy; CSLM, confocal scanning laser microscopy; SDS, sodium dodecyl sulfate; L_n, number-average contour length; L_w, weight-average contour length; $\langle R_{ee}^2\rangle^{1/2}$, root-mean-square end-to-end distance; L_p, persistence length; M_w, weight-average molecular weight; M_n, number-average molecular weight.

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