# Comparative Study on Structural Features of $\alpha$ Chitin from *Xiphopenaeus kroyeri* and Its Precipitated Product from Phosphoric Acid Solution

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**ABSTRACT:** A sample of $\alpha$ chitin was obtained from shells of the shrimp *Xiphopenaeus kroyeri* by demineralization and deproteinization processes. This sample of native $\alpha$ chitin (NC) was dissolved in concentrated phosphoric acid and recovered by precipitation in an alkaline aqueous solution, giving rise to a sample named PC. Solid-state carbon-13 nuclear magnetic resonance ($^{13}$C-NMR), X-ray diffraction, and atomic force microscopy (AFM) techniques were used to compare both samples and to investigate structural changes in $\alpha$ chitin under the action of phosphoric acid. $^{13}$C-NMR experiments and X-ray diffraction results revealed a high crystalline order in both samples. Structural differences were due to the higher distance between adjacent chains along the *b* axis and the consequent decrease in the number of intermolecular hydrogen bonds in the sample recovered from solution. While remarkable supramolecular differences could be observed in the fibrillar crystals of the NC sample, the PC sample was characterized by only one type of morphology. © 2002 John Wiley & Sons, Inc. J Appl Polym Sci 83: 151–159, 2002

**Key words:** crystallization; solid-state structure; atomic force microscopy (AFM)

## INTRODUCTION

Chitin is a linear polysaccharide, composed of $\beta$-(1→4)-2-deoxy-2-acetamido-D-glucopyranose repeating units. It is similar to cellulose from the viewpoint of both its structure and abundance in nature. Chitin is usually found in the supporting tissues of invertebrate animals, fungi, and bacteria.[1] In the native state, this biopolymer occurs in association with proteins, pigments, lipids, and inorganic substances[2] and is chiefly found as a fibrillar semicrystalline material. Chitin is a specially attractive polymer because of its potential applicability in many fields such as in separation membranes, chelating agents, cosmetics, and biodegradable and biomedical materials.[3] Therefore, the dissolution of chitin, which is sparingly soluble in common organic solvents, and the proper-

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ties of its precipitated products, either in solution[4] or in the solid state,[5] have deserved attention.

Three polymorphic forms, namely, $\alpha$, $\beta$, and $\gamma$, have been proposed to natural chitins, to account for X-ray diffraction results.[2] $\alpha$ Chitin is the most abundant and stable polymorph, whereas $\gamma$ chitin is the least characterized. Although rarely found in nature, anhydrous $\beta$ chitin crystallography is well established, with chains packed in parallel.[6–8] Conversely, in $\alpha$ chitin, the chains are arranged in an antiparallel fashion.[9–11]

Different techniques have been used to study the crystal structure and morphology of polymers. High-resolution $^{13}$C-NMR spectra of solid polymeric materials can be obtained with the combination of techniques known as cross polarizaton (CP) and magic-angle spinning (MAS).[12] The chemical shifts of these spectra may be used for structural elucidation in terms of both molecular and crystal structures.[13] In general, while noncrystalline polymers give broad resonances, crystalline compounds give rise to much narrower resonances due to the regularity of the crystalline local environment. CPMAS $^{13}$C-NMR spectra of a variety of $\alpha$ and $\beta$ chitin samples[5,14,15] and precipitated products from *N,N*-dimethylacetamide/LiCl $\alpha$ and $\beta$ chitin solutions[5] have been reported in the literature. Changes in the molecular and segmental mobilities may be evaluated by comparing proton spin-lattice relaxation times in the rotation frame, $T_{1\rho^H}$. This parameter, obtained from the $^{13}$C intensity decays of the resolved peaks during variable contact-time experiments, is sensitive to motional processes in the 10–60 kHz region.[16] To the present, the use of this parameter to elucidate structural changes between different chitin samples has not been reported.

Native $\alpha$ chitin systems have been visualized by diffraction contrast transmission electron microscopy as dark units embedded in a white nondiffracting matrix.[17] Chitin crystals 3 nm in width were reported to be grouped in bundles with an average diameter of 100 nm in the principal layer of the crab *Carcinus maenas*.[17]

Atomic force microscopy (AFM) has been used with success to image the surface morphology of a variety of materials, especially soft biopolymers,[18–23] in the micrometer domain. This technique has the advantage of preserving the molecular features, making possible the nondestructive imaging of these surfaces, without special sample preparations.

In the present work, shells of the shrimp *Xiphopenaeus kroyeri* were demineralized and deproteinized to give native $\alpha$ chitin (NC) films. Part of this product was dissolved in concentrated phosphoric acid at room temperature and recovered in alkali, giving rise to a powdered precipitate (PC sample). In general terms, different processing treatments of semicrystalline polymers produce different properties from the same material and this is reflected by the resulting morphologies. Solid-state $^{13}$C-NMR experiments, X-ray diffraction, and AFM were used to analyze both samples and to investigate the differences between them in relation to their structural order and morphology.

## EXPERIMENTAL

### Materials

The NC sample was isolated from shells of adult *X. kroyeri*, after demineralization and deproteinization. For demineralization, the shells were treated with 0.6*N* HCl for 24 h and washed with water until reaching neutral pH. For deproteinization, the resulting material was treated with 1.25*N* NaOH for 2 h at 95ºC and washed with water until neutral pH. The above procedure was repeated twice. The product was washed with water and dried in an oven at 50ºC. Residual pigments were removed by immersing the sample in ethyl alcohol and acetone for 3–4 days.

The NC sample (10 g/l) was milled and dissolved by stirring in 85% phosphoric acid at room temperature for 30 min. The viscous solution was centrifuged at 8000 rpm for 30 min at room temperature. The solution was precipitated in cold 0.1 N NaOH as a white powder, which was washed with water and ethyl alcohol, and dried at 60°C.

### Methods

#### *$^{13}$C-NMR Spectroscopy*

Solid state $^{13}$C-NMR experiments were carried out with a Varian Inova 300 spectrometer operating at a $^{1}$H frequency of 299.9 MHz and a $^{13}$C frequency of 75.4 MHz. All experiments were performed at probe ambient temperature using gated high-power decoupling. A zirconium oxide rotor of 7 mm diameter with a Kel-F end cap was used to acquire the NMR spectra at rates of 6 kHz. The $^{13}$C-NMR spectra were referenced to the chemical shift of the methyl carbons of hexamethylbenzene

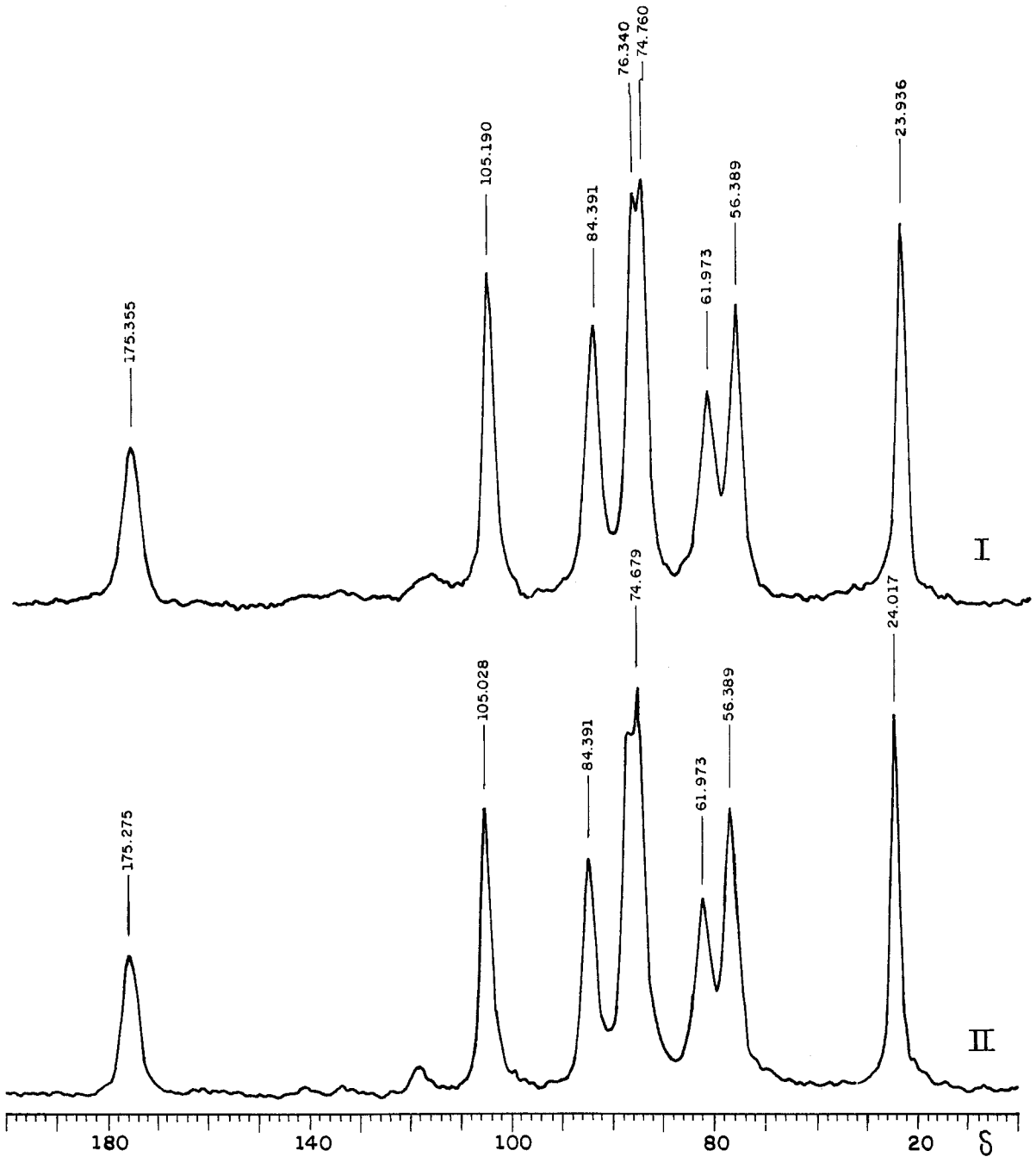


**Figure 1** CPMAS $^{13}$C-NMR spectra acquired at a contact time of 800 μs; trace I, native α chitin (NC sample), and trace II, product precipitated from phosphoric acid solution (PC).

(17.3 δ). These $^{13}$C-NMR spectra were obtained by CPMAS with 2 s of delay. Variable contact times experiments were performed at contact times in the range 200–8000 μm. The $T_{1\rho^H}$ values were determined from the intensity decay of the peaks with increasing contact times.

### *X-ray Diffraction*

X-ray diffraction diagrams of the NC and PC samples were obtained by the powder method with a Rigaku diffractometer Model DMAX 2200. Diffractograms were recorded in the reflection mode in the angular range 5–50° (2θ), at ambient temper-

**Table I Chemical Shifts ($\delta$) and $T_{1\rho}^{H}$ Values for Carbon Atoms of the NC and PC Samples**

| | ($\delta$); $T_{1\rho}^{H}$ (ms) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | C═O | C1 | C4 | C5 | C3 | C6 | C2 | —$CH_3$ |
| NC | (175.3) | (105.1) | (84.3) | (76.5) | (74.7) | (61.9) | (56.4) | (23.9) |
| | 7.3 | 7.0 | 6.5 | 5.6 | 10.1 | 5.5 | 2.2 | — |
| PC | (175.2) | (105.0) | (84.3) | (76.7) | (74.6) | (61.9) | (56.3) | (24.0) |
| | 6.1 | 6.7 | 6.8 | — | 6.9 | 8.9 | 6.7 | — |

ature, using CuK$\alpha$ radiation (0.1542 nm), generated at 40 kV and 40 mA, and a graphite secondary monochromator to select the $K_{\alpha 1}$ $K_{\alpha 2}$ doublet.

### *AFM*

A Topometrix TMX 2010 Discoverer (Topometrix) instrument, equipped with an AC mode AFM probe head and a 70-$\mu$m Tripot scanner, was used to obtain scanning probe microscope images. The tips (Topometrix 1660$^{TM}$) were made of Si, with a spring constant of about 40 N/m and resonance frequencies in the 100–150 kHz range. Chitin samples were fixed on double-sided adhesive tape and the AFM images were obtained in air. Changes in the sample vertical position are presented as a height image. Changes in the phase angle of probe oscillation are presented as phase images.

## RESULTS AND DISCUSSION

The treatment applied to the shells of the shrimp *X. kroyeri* gave rise to films of $\alpha$ chitin, used in the solid-state $^{13}$C-NMR and AFM experiments. This same native sample (NC) was milled to obtain X-ray diffraction data. The milled NC sample was dissolved in phosphoric acid and recovered as a powdered precipitate, following the procedure reported in the literature.[4] According to this method, after 1 h in the phosphoric acid solution, the precipitated product is not modified chemically and its molecular weight suffers only a small reduction.[4]

CPMAS $^{13}$C-NMR spectra, acquired at a contact time of 800 $\mu$s, of NC and its precipitated product from the phosphoric acid solution (PC) are shown in Figure 1. Both traces, trace I obtained for NC and trace II obtained for PC, reveal eight totally or partially resolved peaks, attributed to the chemically different carbon atoms of $\alpha$ chitin.[14] The NMR lines are symmetric and narrower than are those reported in the literature for crab and lobster $\alpha$ chitin,[15] which reflects the possibly higher crystallinity of the NC and PC samples. Moreover, no broad component may be seen in the spectra of Figure 1. According to Table I, no significant variation in chemical shifts is

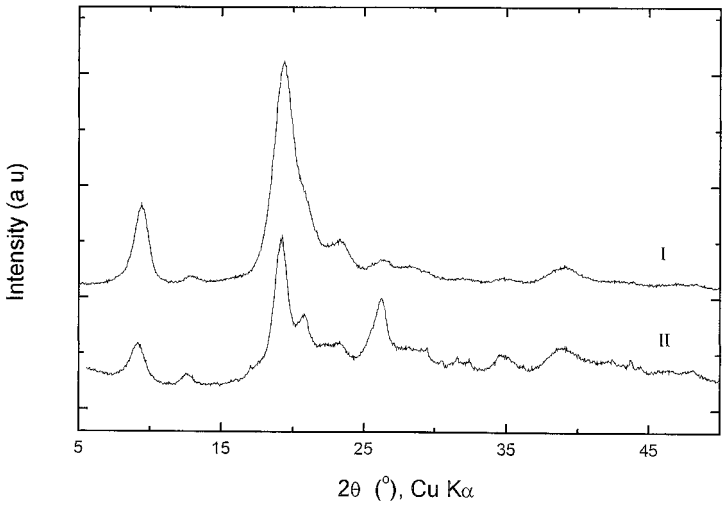


**Figure 2** X-ray diffractograms of native $\alpha$ chitin (NC sample), trace I, and the precipitated product from phosphoric acid solution (PC sample), trace II.

**Table II Crystallographic Parameters of NC α Chitin**

| (*hkl*) | 2θ (°) | *d*-Spacing (nm) | Intensity (%) |
|---|---|---|---|
| (020) | 9.40 | 0.940 | 52.3 |
| (021) | 12.85 | 0.688 | 4.7 |
| (110) | 19.40 | 0.457 | 100 |
| (130) | 23.31 | 0.381 | 10.9 |
| (112) | 26.20 | 0.340 | 6.8 |
| (060) | 28.60 | 0.312 | 2.3 |
| (142) | 31.94 | 0.280 | 1.9 |
| (152) | 34.83 | 0.257 | 3.1 |
| (063) | 39.05 | 0.230 | 6.9 |
| (171) | 39.59 | 0.227 | 3.1 |

observed for the corresponding peaks of the two samples. The chemical shifts of C1 and C4 carbon atoms in 1,4-linked carbohydrates are known to be sensitive to the conformation of the glycosidic linkage.[24] Since there is no difference in those parameters, it may be concluded that the conformation of the glycosidic linkage is maintained after dissolution and precipitation.

Table I also shows the values of the proton spin-lattice relaxation time in a rotation frame $T_{1\rho^H}$, determined from the slopes of the peak intensities as a function of contact time, from 200 to 8000 μs. As expected, $T_{1\rho^H}$ values for C1 and C4 practically do not change for the two samples. Although C═O is supposed to be involved in intra- and intermolecular bonds, the values of $T_{1\rho^H}$ determined for the carbonyl carbons of the samples do not vary significantly. Greater variations were observed among $T_{1\rho^H}$ values determined for C2, C3, and C6.

According to the model proposed by Minke and Blackwell,[10] following wide-angle X-ray diffraction studies on a native highly crystalline α chitin, the hydroxyl group linked to C3 is oriented to form an intramolecular bond with the oxygen atom of an adjacent glycosidic ring, of the type (O-3′-H · · · O-5). The present results show a significant decrease in $T_{1\rho^H}$, determined for C3 in the PC sample. This change seems to indicate the contribution of that interaction to the higher rigidity of C3 in the PC sample, compared to NC.

The $T_{1\rho^H}$ value determined for C2, the carbon atom covalently linked to the acetamide group, indicates a higher mobility for C2 and, consequently, for the acetamide group, in the the PC sample. The same trend was observed for C6. The higher value of $T_{1\rho^H}$ = 8.9 ms, determined for C6 in PC, compared to $T_{1\rho^H}$ = 5.5 ms, determined for the same carbon atom in NC, may be reflecting the specific interactions of the primary hydroxyl groups, covalently linked to C6. In NC, the —$CH_2$OH groups are involved in intermolecular hydrogen bonds [10] and, therefore, are supposed to contribute to the higher rigidity of C6. The significant changes in the $T_{1\rho^H}$ values determined for C2 and C6 suggest a decrease in intermolecular hydrogen bonds for the PC sample.

Figure 2 shows X-ray diffractograms of NC and PC samples. The diffraction parameters are summarized in Tables II and III. Both samples present a high crystalline order. This assertion can be supported, since the occurrence of the (001) reflection was not observed. The intensity of this reflection is known to decrease as the crystallite size increases and is completely eliminated when larger crystallites are formed.[10]

Changes in the X-ray pattern may be observed for the two samples. Although the two patterns are similar to each other in terms of the position of the reflections, the relative intensities are rather different. The peak at 2θ = 9.40°, associated with the plane (020) and with the most ordered regions involving the acetamide groups,[5] presents a higher intensity in the NC sample than does the same peak in the precipitated product (PC). In the diffractogram of NC, a strong peak appears at 2θ = 19.40°. This peak, which is associated with the plane (110), was observed as the strongest meridional reflection for a highly crystalline sample of α chitin, obtained from the mandibular tendon of the lobster *Homarus americanus*.[10] In the diffractogram of PC, the same peak appears as the one with the highest intensity. In the same diffractogram, two reflections deserve notice: the peaks at 2θ = 20.80° and at 2θ = 26.25°, associated, respectively, with the planes (111) and (013). For the NC sample, the data

**Table III Crystallographic Parameters of PC α Chitin**

| (*hkl*) | 2θ (°) | *d*-Spacing (nm) | Intensity (%) |
|---|---|---|---|
| 020 | 9.15 | 0.966 | 33.7 |
| 021 | 12.55 | 0.704 | 9.3 |
| 110 | 19.25 | 0.461 | 100 |
| 111 | 20.80 | 0.427 | 12.3 |
| 121 | 22.29 | 0.398 | 0.1 |
| 130 | 23.21 | 0.383 | 7.1 |
| 013 | 26.25 | 0.339 | 47.3 |
| 152 | 34.55 | 0.259 | 14.8 |
| 211 | 38.85 | 0.232 | 11.9 |

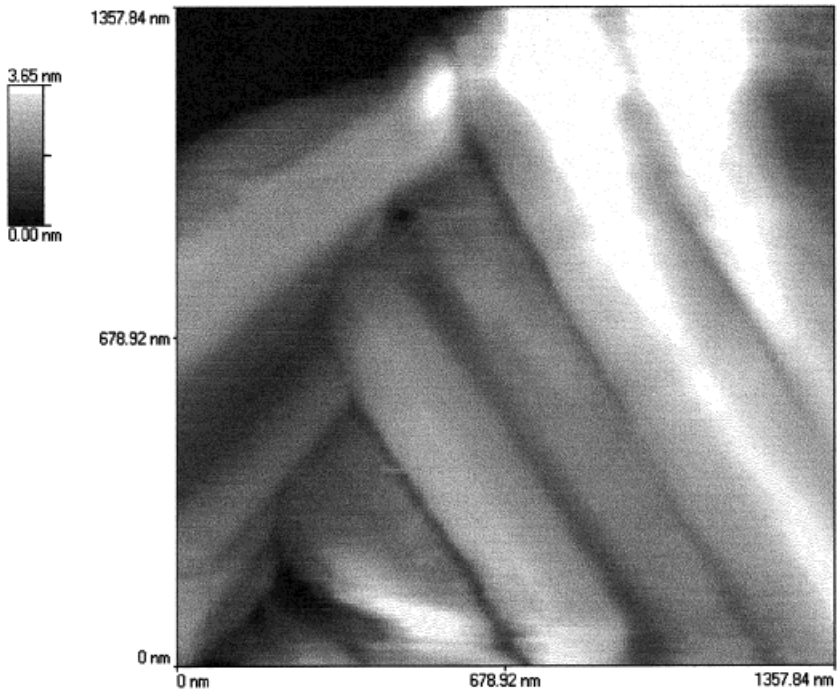


**Figure 3** AFM topographic image of $\alpha$ chitin assembly imaged by scanning the rostrum (NC sample).

indicate that there is a preferred orientation of the crystallites, since the equatorial reflections ($hk0$) are the most intense. In contrast, for the PC sample, the crystallites seem to have no preferred orientation.

Considering an orthorhombic unit cell, with a $P2_12_12_1$ space group, as reported in the literature,[10] the unit cell parameters were determined to be $a = 0.476 \pm 0.003$ nm, $b = 1.876 \pm 0.007$ nm, and $c = 1.023 \pm 0.007$ nm for the NC sample and $a = 0.479 \pm 0.001$ nm, $b = 1.921 \pm 0.008$ nm, and $c = 1.035 \pm 0.001$ nm for the PC sample. The parameters determined for the NC sample are very close to those reported in the literature for the deproteinized lobster apodemes.[9] Comparing the data obtained for the NC and PC samples, no significant changes in the $a$ and $c$ parameters are detected, which reveals no conformational differences along the chain axis and in the packing of the parallel chains within a stack (chains along the $a$ axis). Contrarily, the increase in the $b$ dimension of the unit cell of the PC sample indicates that a greater distance separates the adjacent antiparallel stacks. This result is in accordance with the dynamic properties of the solid polymers, given by the $^{13}$C-NMR experiments, in which an increase in the mobilities of C2 and C6 was detected for the PC sample, in relation to NC.

AFM was used to image the external surface of the NC films, namely, the exocuticle of the shrimp carapace, and inner surfaces disclosed by splitting the cuticle fragments into thinner films. In general, NC appears as microfibrils, arranged differently, depending on the surface being probed. Crystals of great thickness (200–300 nm), arranged in a peculiar architecture, may be observed in Figure 3, a topographic image obtained by scanning a particular piece of the NC film, called a rostrum, which constitutes the spinelike prolongation of the carapace. According to the literature[17] and because of their dimensions, these crystals might consist of bundles of grouped highly crystalline microfibrils, not resolved with the experimental conditions used. Similar crystals, not shown in the present work, have been scanned in other regions of the exocuticle.[25]

Crystals of lower crystallinity can also be found, mainly on other parts of the carapace. Figure 4 presents fibrillar arrangements observed by scanning the NC sample. In Figure 4(a), nearly straight and aligned microfibrils can be visualized, and in Figure 4(b), the microfibrils are arranged in a more disordered way. In both images, the typical size of the fibrils varies from 30 to 90 nm.

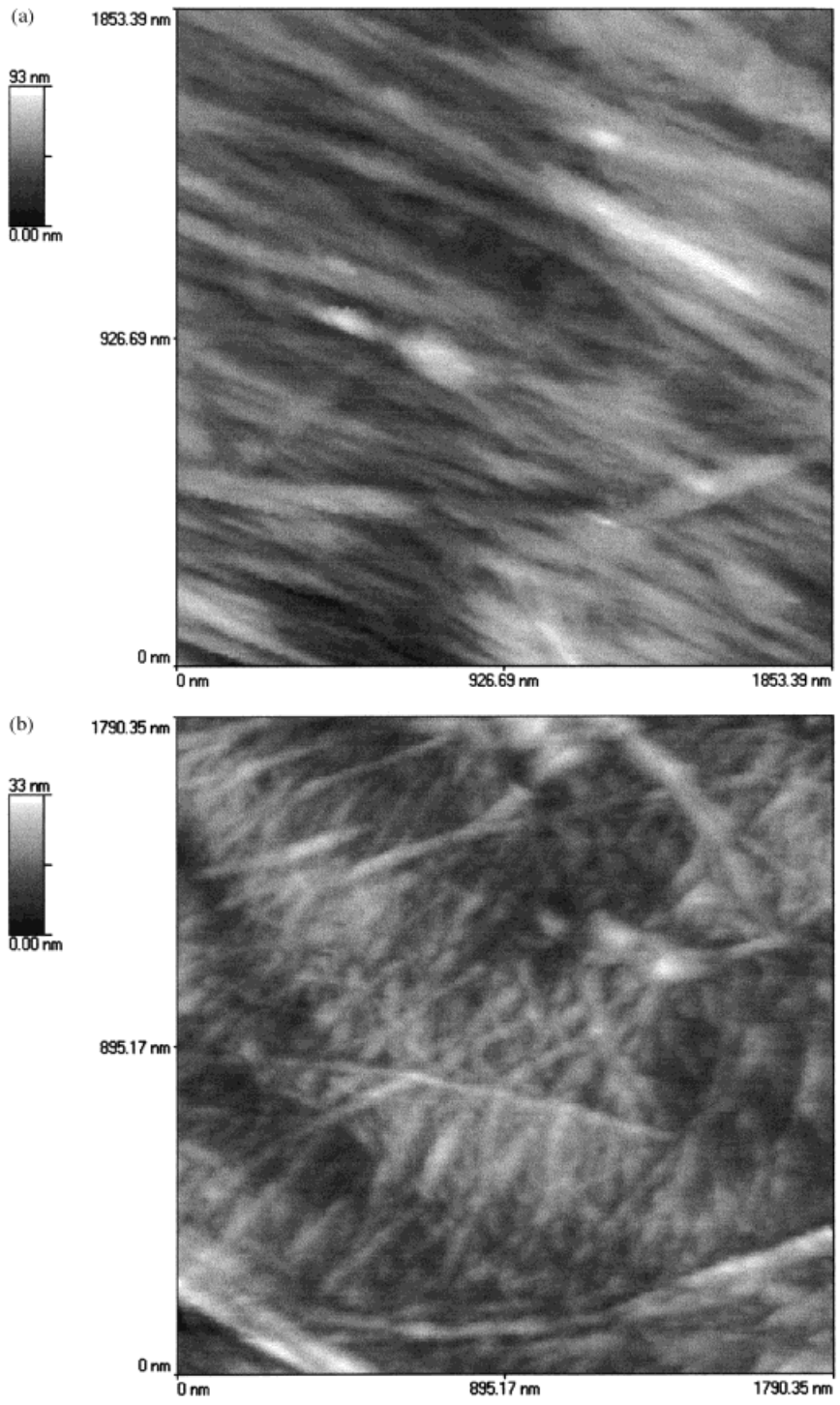


**Figure 4** AFM topographic images of α chitin microfibrils in the NC sample: (a) nearly straight and aligned microfibrils; (b) nonstraight and nonaligned microfibrils.

While the previous figures revealed remarkable supramolecular differences in α chitin microfibrils occurring in the NC films, only one type of morphology was observed by scanning the PC sample. Figure 5(a,b) shows a topographic image and the corresponding phase image of the PC powder, respectively, where a granular structure substitutes the fibrillar crystals previously im-

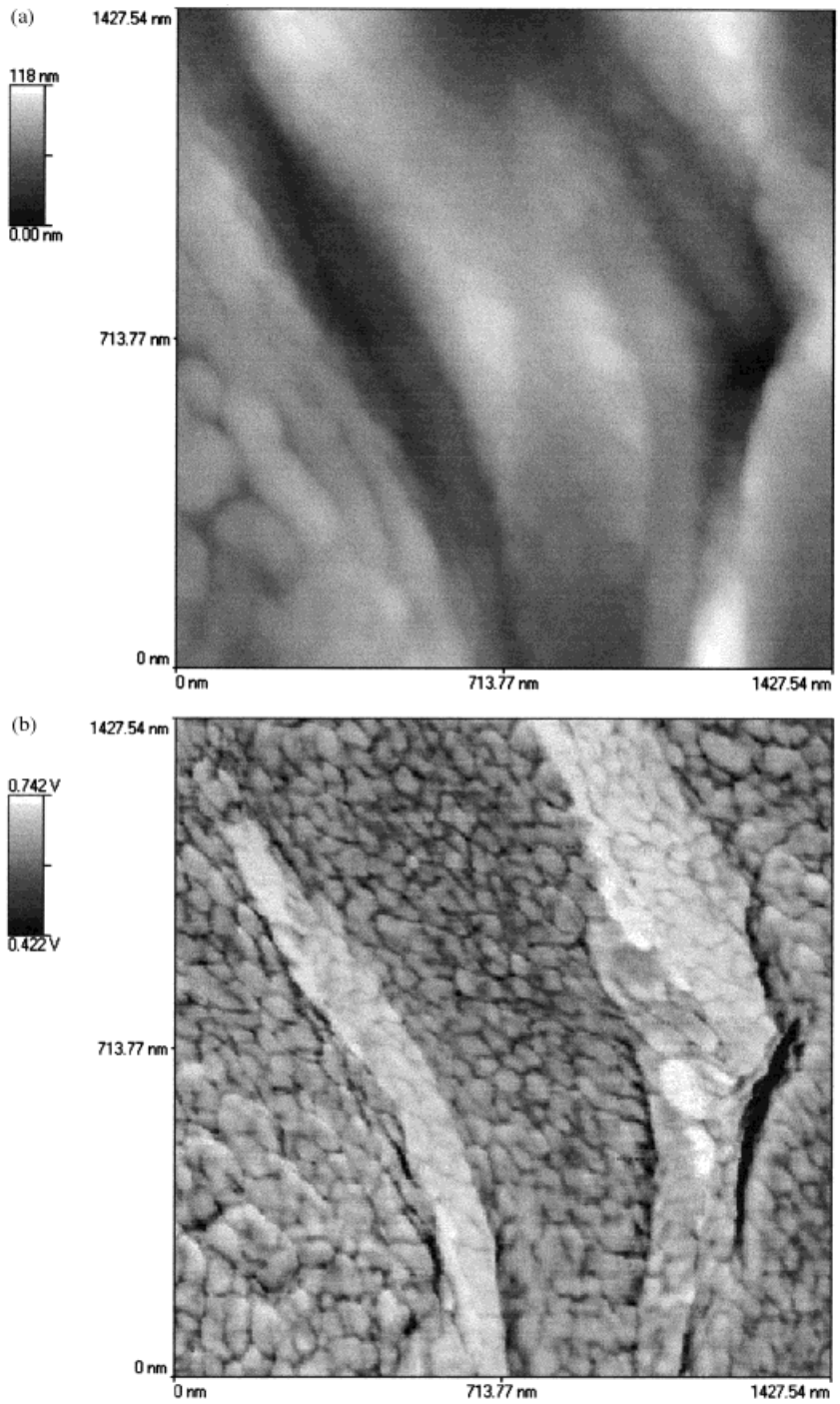


**Figure 5** AFM images of the PC sample: (a) topographic image; (b) phase image.

aged for the NC sample. In the PC sample, large valleys, more than 200 nm in depth, are revealed in the topographic image. These large corrugations of the surface make it difficult to access the local microstructure of the sample. Phase-contrast imaging is known as a powerful method for resolving fine details of rough surfaces[26] and was used to provide information on the morphology of the PC sample. In Figure 5(b), the grain boundaries can be clearly observed and allow the measurement of the crystal size as varying from 30 to 70 nm.

## CONCLUSIONS

The narrow lines in the CPMAS $^{13}$C-NMR spectra and the X-ray diffractograms point to the high crystalline order of α chitin from *X. kroyeri* and its precipitated product from a phosphoric acid solution. The proton spin-lattice relaxation times in the rotation frame, $T_{1\rho^H}$, proved to be a parameter capable of explaining the structural differences appointed by the unit cell parameters. Characteristic morphological features that distinguish the NC films from the PC powder could be visualized by AFM. The fibrillar nature of α chitin in the NC sample and the nonpreferential crystalline arrangement in the PC sample were shown by X-ray diffraction results and corroborated by the AFM data.

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