Honeycomb-Like Architecture Produced by Living Bacteria, *Gluconacetobacter xylinus*

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ABSTRACT

Bacterial cellulose (BC)-producing bacterium, *Gluconacetobacter xylinus* (ATCC53582), was found to move along linear microgrooves of a stripe-patterned cellulosic scaffold. On the basis of this finding, fabrication of honeycomb-patterned BC was attempted by controlling the bacterial movement using a agarose film scaffold with honeycomb-patterned grooves (concave type). The patterned agarose film was prepared by three steps. The first was transcription of a honeycomb-patterned polycaprolactone film template with polydimethyl siloxane. When the bacteria were cultured on the scaffold under atmospheric conditions, only bacterial proliferation was observed. Honeycomb-patterned BC was obtained when cultured under a humid CO_2 atmosphere. Electron diffraction and polarized microscopic observation showed that the patterned BC comprised of the well defined cellulose Iα microfibrils.

As another attempt to fabricate honeycomb–patterned BC, the bacteria were cultured on the patterned cellulose and agarose film with convex type of honeycomb. This culture yielded no honeycomb-patterned BC. Therefore, concave type honeycomb scaffold is more suitable to fabiricate honeycomb-patterned BC.

Keywords

Cellulose Iα, Electron diffraction, Honeycomb- patterned bacterial cellulose, Self-organization,

Introduction

Bacterial cellulose (BC) produced by some bacteria has been widely studied as a feedstock for functional materials due to its higher purity, mechanical strength and biodegradability as compared with plant cellulose (Yano et al. 2005). Recently, BC has been investigated as a potential scaffold for tissue

engineering (Bäckdahl et al. 2006; Svensson et al. 2005). Mesoscopically patterned films, in particular honeycomb-patterned films with submicron to micron pore size are also of particular interest for tissue engineering as well as potential application in molecular separation and biointerface (Tanaka, Takebayashi, Miyama, Nishida and Shimomura 2004; Fukuhira et al. 2006; Tsuruma, Tanaka, Fukushima and Shimomura 2005). Honeycomb patterned materials have architectures that are lightweight and have high mechanical strength. Therefore, the transformation of BC into honeycomb-patterned materials may further expand the novel utilization of BC and develop novel functional biomaterials.

Honeycomb-patterned films are easily fabricated from amphiphilic polymers by self-organization during solvent casting under humid air conditions (Widawski, Rawiso and François 1994; Karthaus, Maruyama, Cieren, Shimomura, Hasegawa and Hashimoto 2000). Honeycomb-patterned cellulose films have been fabricated by a similar method using a cellulose triacetate chloroform solution (Kasai and Kondo 2004; Nemoto et al. 2005), unfortunately the resulting honeycomb pore size distribution was not uniform. Honeycomb-patterned cellulose films with uniform pores was prepared by a transcription method Nemoto et al. 2005; Yabu and Shimomura 2005), however this utilized regenerated cellulose and not BC. All of these fabrication techniques utilize polymer solutions. When BC is dissolved in a suitable solvent it is transformed into regenerated cellulose, leading to the loss of BC characteristics. Thus, a novel technique for fabrication of honeycomb-patterned BC should be developed.

Gluconacetobaqcter xylinus (old nomenclature, *Acetobacter xylinum*)is a cellulose secreting bacteria that is propelled by the secretion of BC.[12,13] The secreted cellulose is randomly deposited behind the moving micro-organism to produce a gelatinous mat this is used as “nata de coco” in the food industry in south-east Asia. We hypothesize that the direction of bacterial movement determines the orientation of the deposited BC. On the basis of the hypothesis, two strategies were proposed for the fabrication of honeycomb-patterned BC by controlling bacterial movement. In the first strategy, a convex type honeycomb-patterned regenerated cellulose film was used as the scaffold. As *G.xylinus* has been reported to move along the cellulose rail while secreting BC,[13] it is expected that the bacterium might

move alongside the convex honeycomb or ridge of honeycomb-shaped mountain range. In the other strategy, a concave type agarose film scaffold was used. This strategy was made based on the expectation that the bacterium would move physically along the valley of honeycomb pattern. In this paper we report on the fabrication of BC honeycomb films using these two strategies.

Experimental

Materials

A copolymer (CAP) was synthesized with dodecylacrylamide and ω-carboxyhexylacrylamide as reported previously.[14] Polydimethylsiloxane (PDMS) and its curing agent (SYLGARD 184 Silicone Elastomer Kit) were produced by Dow Corning Corp. Midland, MI. All chemicals with reagent grade were purchased from Wako Pure Chemicals, Osaka, Japan.

Fabrication of polymeric scaffold

A fabrication scheme for convex and concave type honeycomb-patterned films is shown in Figure 1. The first template was a honeycomb-patterned film fabricated with a mixture of polycaprolactone and CAP (weight ratio, 9:1).[14,15] This was a convex film having honeycomb-patterned walls. The second template was made by pouring PDMS into the first template together with a curing agent.[15] After curing at room temperature for 48 h, the resultant elastomer (second template) was peeled off the first template. The second template was then used to make the convex cellulose and agar films. For the convex regenerated cellulose films the second template was pressed onto 5 mg/mL of a cellulose triacetate/chloroform solution. After evaporating the chloroform, the cellulose triacetate film was peeled from the template and saponified with 2.5% of sodium methoxide in methanol overnight at room temperature. Similarly, the second template was pressed onto a hot agarose aqueous solution (1.5%), then cooled and the convex-type of agarose film was peeled from the template.

The third template with a convex honeycomb pattern was prepared by transcription of the second PDMS template with new PDMS. The resultant third PDMS template was stamped onto hot agarose containing nutrients from the Hestrin-Schramm (HS) liquid medium (0.5% yeast extract, 0.5% polypeptone, 2.0% glucose, 0.12% citric acid and 0.27% Na_2HPO_4), [16] and the concave agarose film with honeycomb-patterned microgrooves was obtained after cooling.

A stripe-patterned cellulose film was prepared in accordance with previous reports.[10,17]

Culture of bacteria

The bacterium was pre-cultured in the HS medium for about 4 days. Twenty microliters of the culture medium was inoculated onto the stripe-patterned cellulose placed on a glass plate. The bacterial movement was observed on an optical microscope (Compound light microscope BH-2, OLYMPUS)

The pre-cultured medium was inoculated onto the convex and concave-type honeycomb-patterned films and placed on a flat agarose gel as a support. The surface of the patterned films was observed using an optical microscope and a scanning electron microscope after 4 days culture. When using the patterned agaraose films, two kinds of supporting gels were prepared. One contained nutrients from the HS medium, the other one did not. The bacteria were incubated for 2-4 days at 28^{o}C under several conditions with variations of humidity and gas atmospheres. The conditions were made by floating petri-dish for the culture on the water in a chamber that equipped two tubes for gas inlet and outlet, and monitors for humidity, temperature, oxygen (GOA-6H-S, GASTEC Co., Ayase, Japan) and CO_2 (Cosmotector XP-314, New Cosmos Electric Co., Osaka, Japan).

Scanning electron microscopic (SEM) observation

A specimen was fixed with OsO_4, followed by dehydration with successive ethanol exchange (20-99.5% ethanol) and finally critical point dried with CO_2. SEM images were observed on a JSM-6301F Field-emission SEM (JEOL, Tokyo, Japan) after ion-spattering with platinum-palladium. The accelerator voltage was 5.0 kV.

FT-IR microscopy and electron microscopy

The honeycomb-patterned BC on the agarose scaffold was immersed in hot water, and washed several times with hot water. After drying the BC *in vacuo,* a part of resultant film was placed on a copper grid for transmission electron microscopy. First FT-IR spectra were measured on a Perkin Elmer Spectrum One equipped with a microscope attachment. Spectra were recorded by an MCT detector, accumulating 128 scan from 4000 - 700 cm^{-1} with a resolution of 4 cm^{-1}. The aperture was 50 μm x 50 μm.

The micrographs and electron diffraction diagrams were taken with a JEOL-2000EX II TEM operated at 100 kV, and recorded on Mitsubishi MEM film. Low dose defocused imaging in the bright field mode was used to visualize the sample without further contrast enhancement. The images were taken at 2500 - 6000x magnification. The electron diffraction diagrams were obtained from highly oriented regions of the spiral array of bacterial cellulose microfibrils by an electron probe of approximately 500 nm. With this condition, nearly perfect fiber diffraction patterns were recorded with an exposure of 2.8 sec at a camera length of 15 cm.

Results and Discussion

Culture of *G. xylinus* on convex type honeycomb-patterned films

As *G. xylinus* has been reported to move along cellulose rails while secreting BC,[13] we first attempted to fabricate honeycomb-patterned BC with pores of micron order by culturing *G. xylinus* on the convex type honeycomb-patterned cellulose film (first strategy). No deposition of bacteria or cellulose on the top of the scaffold or wall of the honeycomb pattern was observed after 4 days culture (bacteria were subcultured in the HS medium prior to inoculation on the scaffold film). However, coil-like depositions on the surface of supporting agarose were observed along the inside of the wall of the honeycomb-patterned film, as shown in Figure 2 (A). A similar phenomenon was observed in the culture with the convex type honeycomb-patterned agarose film [Figure 2 (B)]. Based on the

morphology it is likely that the deposition is mainly comprised of bacterial body and a small amount of cellulose. These results suggested that no honeycomb-patterned BC was prepared by the method based on the first strategy. However, the bacteria might recognize wall with the recognition being independent of scaffold material. Therefore, the coil-like proliferation of *G. xylinus* and subsequent BC production seems to be brought about by physical control.

Culture of *G. xylinus* on stripe-patterned cellulose

Based on the above findings it was assumed that the bacteria would move along a microgroove patterned film. Bacterial movement along microgrooves was investigated using a simple stripe-patterned film scaffold. Striped, microdotted, laddered as well as honeycomb-patterned polymeric films are readily prepared by a self-organization film casting process.[17] Using cellulose triacetate in chloroform we have produced stripe-patterned regenerated cellulose scaffolds.[10] The movement of *G. xylinus* was optically monitored after the bacteria in HS medium was inoculated on the patterned film. The *G. xylinus* was observed to move along the microgrooves of the striped scaffold; no movement was observed along the apex or ridge of the grooves (Figure 3, and Appendix 1). This suggests that the motion of *G. xylinus* can be controlled using microgooves. However, as the cellulose film did not contain any nutrients significant cellulose deposition on this scaffold was not observed.

Culture of *G. xylinus* in concave type of honeycomb-patterned agarose film

To facilitate cellulose deposition a patterned polymeric film was prepared from agarose containing the nutrients from the HS medium. The honeycomb-patterned agarose scaffold was prepared by the transcription process outlined in Fig. 1.[10,15] Utilizing a series of three transcription processes a concave type honeycomb-patterned agarose gel scaffold was produced. Included in Fig. 1 is a scanning electron microscopic (SEM) image of the resultant agarose gel.

Figure 4 shows the results obtained when *G. xylinus* was inoculated on the agarose scaffold, which was placed on a flat support of agarose, at 28°C under atmospheric conditions. Honeycomb-like

deposits were observed over the entire medium when both the scaffold and the supporting agarose gel contained nutrients from the HS medium [Figure 4 (A)]. However, the area of deposition was limited when both the scaffold and supporting medium lacked nutrients, or when only the patterned scaffold contained nutrients [Figure 4 (C)]. This suggests that substantial nutrient transport from the supporting agarose gel to the scaffold occurs. Under high magnification only bacteria were observed, the long and slender oval deposits seen in Fig 4(B), no ribbon-like BC were apparent. Thus, the observed deposition resulted from proliferation of bacteria along the microgrooves of the honeycomb pattern, not but from cellulose production.

The low cellulose production was attributed to suppression of bacterial movement under the atmospheric culture conditions. Under these conditions, the moisture content of the scaffold surface decreased as water evaporated from the scaffold impeding bacterial movement. Accordingly, we cultured the bacteria under high-humidity conditions (95% relative humidity, RH) to maintain moisture on the medium surface. Figure 5 (A) shows a SEM image of the surface of the scaffold after being cultured for 2 days. The production of ribbon-like material, BC, was apparent in comparison with that shown in Figure 4 (A), however bacterial proliferation remained active.

In order to improve cellulose production, the culture atmosphere was further changed. Under an oxygen stream and high humidity, neither proliferation nor cellulose production was observed. This confirms that high concentrations of oxygen inhibit its growth despite the fact that *G. xylinus* is an aerobic bacterium. Under a stream of nitrogen containing about 1% oxygen, very little proliferation and cellulose production was also observed. However, when *G. xylinus* was cultured under a stream of carbon dioxide at high humidity (85% CO_2, 1% O_2, 95% RH) for 4 days hexagonal deposits were observed over the entire medium [Figure 5 (B)]. Under high magnification the deposits were found to be cellulose fibrils [Figure 5 (C)]. Each hexagon-like circle contained one or a very few of bacterium, indicated by the arrows in the figure. These results suggested that cellulose production was active under the CO_2-rich conditions, while bacterial proliferation was suppressed.[18] Thus, the fabrication of honeycomb-patterned BC with pores was developed by selecting unexpected culture conditions. We

deduced the BC production on the scaffold from these results as follows. BC deposition on the scaffold resulted from the bacterial movement. In particular, the bacteria smoothly moved on the wet surface similar to the liquid atmosphere and along the larger groove than bacterial size (ca.1 μm x 5 μm). Accordingly, the bacterial motion was independent of the shape of micro-grooves. In fact, the bacteria could move along the linear grooves and hexagonal grooves.

Characterization of honeycomb-patterned BC

The BC structure in the honeycomb pattern was characterized by FT-IR and electron diffraction. The patterned film was easily floated off by dissolving the agarose scaffold in warm water. The thickness of the cellulosic material after 4-days of culture was 1.3 μm, as measured on a 3-D laser microscope (KEYENCE, Japan, Appendix 2). Under the polarizing microscope, the network shows a Maltese-cross extinction pattern, indicating that the crystallites lie along the circumference (tangential or negative birefringent, Appendix 3). The cellulosic nature was unambiguously confirmed from its FT-IR spectra and electron diffraction diagram. In the FTIR spectra shown in Figure 6, the specific band assigned to the cellulose Iα allomorph at 3240 cm^{-1} can be clearly seen as compared with that of normal BC mat, while that of cellulose Iβ at 3270 cm^{-1} is not as distinct.[19,20]

A typical electron diffraction diagram from an oriented region is shown in Figure 7. The diffraction spots are visible up to the ninth layer line, corresponding to a resolution of 0.13 nm. The characteristic diffraction spot in the 3rd layer line (arrow) together with a lack of a medridional spot at the 2nd layer line are the characteristic of the Iα allomorph.[21] Again this analysis indicates qualitatively but not quantitatively, the cellulose networks comprised of Iα–rich cellulose. These results suggest that the obtained honeycomb-patterned network is a continuous porous films built up with highly oriented and crystalline cellulose microfibrils.

The honeycomb pattern formation of BC was summarized as follows., The bacteria follow the grooves of concave honeycomb scaffold, further thickening the surrounding grooves leaving cellulose microfibrils behind them. Since they move by making a series of linked turns, the network can be

formed gradually. At the same time, some bacteria continue to follow the groove of a single hump, thus make closely packed network with spiral patterns (insert in Fig 7). The microfibril aggregation and crystallization occurred in the narrow space of grooves, forming highly oriented and crystalline BC.

Conclusion

We succeeded in controlling the movement of *G. xylinus*, and in establishing a fabrication technique for honeycomb-patterned BC with high orientation, using living bacteria under cultural conditions of high humidity and high CO_2 concentration. This fabrication technique is an example of how microorganisms can be utilized as micromachines for constructing new materials with micro-architecture, and will be applied to the creation of other patterned materials using microorganisms that secrete extracellular matrixes. Thus, we demonstrate a novel approach to material production by combining polymer science and microorganism technology.

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FIGURE CAPTIONS

Figure 1. Preparation scheme of honeycomb-patterned agarose gel as a scaffold for the culture of bacteria.

Figure 2. *G. xylinus* cultured on (A), cellulose and (B), agarose convex type honycomb-patterned films placed on flat agarose gels.

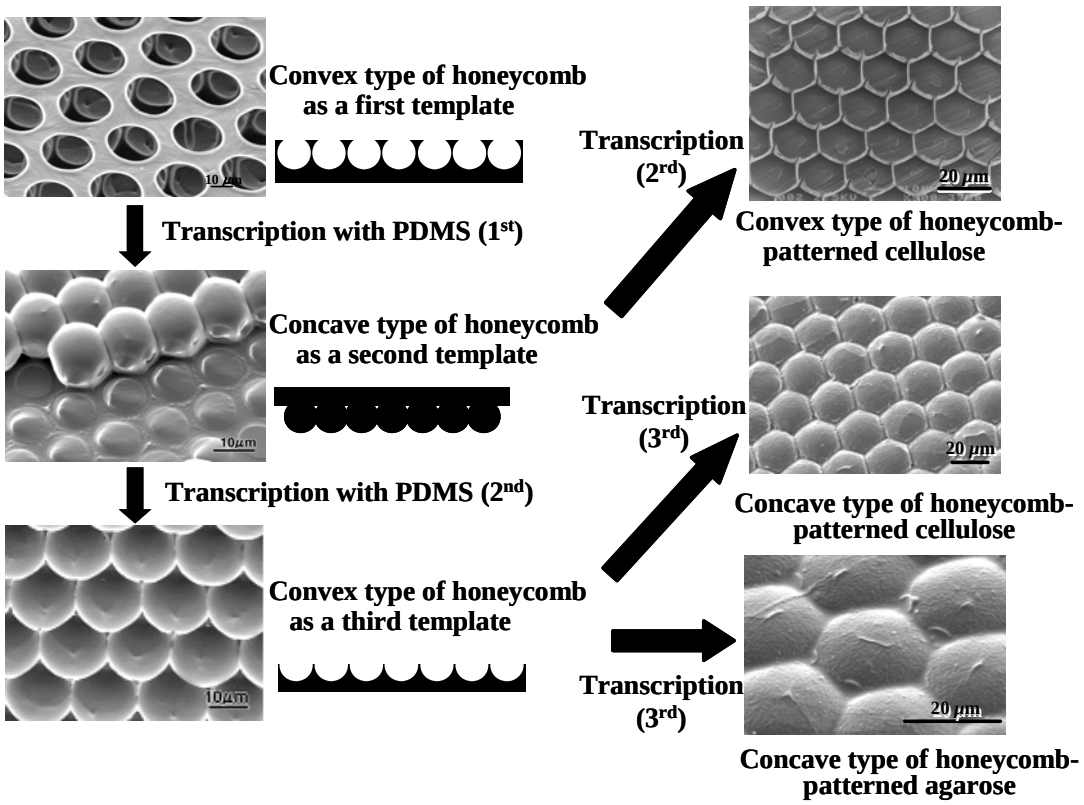
Figure 3. Optical microscopic images for the motion of *G. xylinus* along the linear grooves of stripe-patterned cellulose. Oval; indicates the position of the bacteria, black bars and arrows indicate the initial position of the bacteria. Plate A is an AFM image of the stripe-patterned cellulose film scaffold.

Fig. 4. *G. xylinus* cultured on concave honeycomb-patterned agarose under atmospheric conditions. (A), both patterned gel and supporting gel contained nutrients from HS medium; (B), magnified image of (A); (C), neither patterned agarose nor supporting agarose contained nutrients.

Fig.5. *G. xylinus* cultured on honeycomb-patterned agarose under humid conditions in (A) air and (B) humid CO_2 atmosphere. (C) shows a magnified image of (B). Arrows indicate bacteria.

Figure 6. FT-IR spectra of （A), honeycomb-patterned BC and (B), normal BC as a reference. Each spectrum was monitored in 50 x 50 um area. Insert shows a magnified spectra in the region of 3000-3600 cm^{-1}. The bands at 3240 cm^{-1} is assigned to cellulose Iα.

Figure 7. Electron diffractogram of Honeycomb-patterned BC. Two small arrows indicated asymmetric diffractions, evidencing that the BC was cellulose Iα. Large arrow indicates the 9th layer diffraction corresponding to 0.13 nm resolution. Inserts show transmittance images of the honeycomb patterns.



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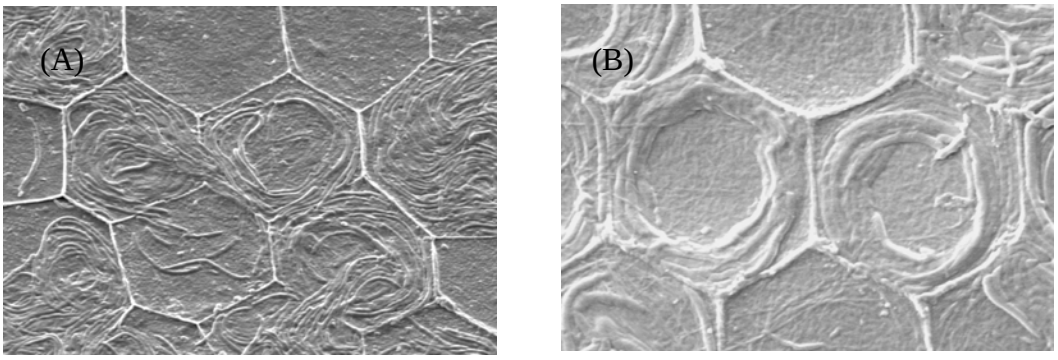

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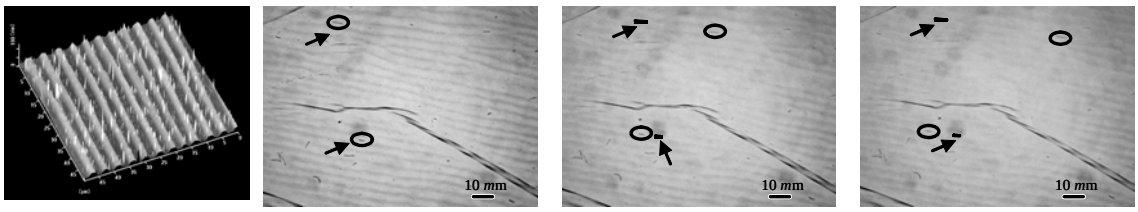

(A) 0 min 30 min 50 min

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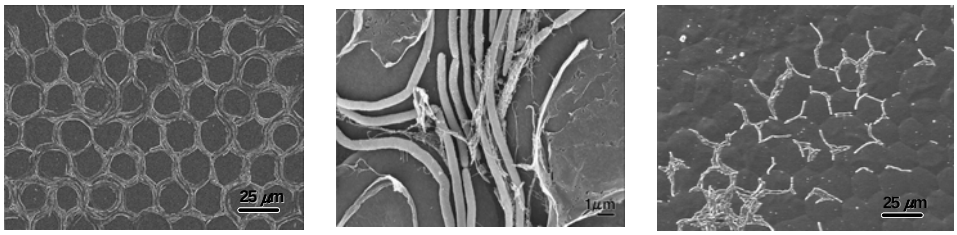

(A) (B) (C)

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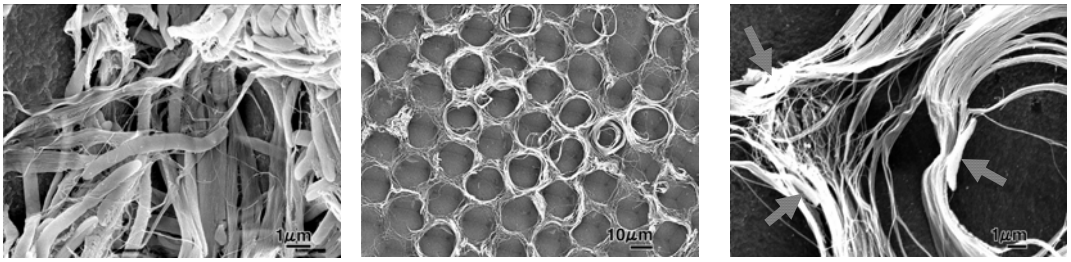

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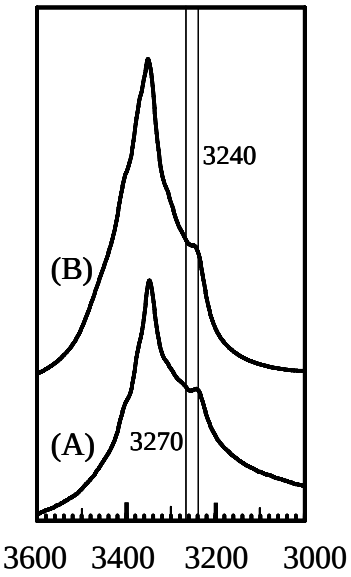

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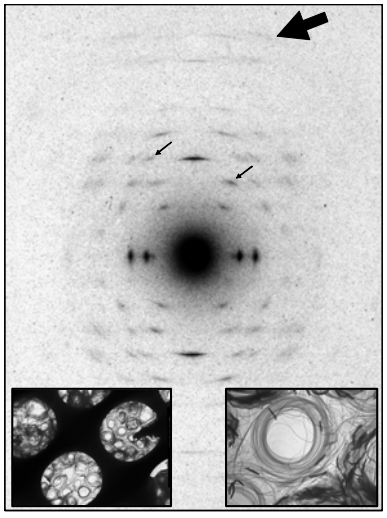

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