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Experiment report

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In situ WAXS monitoring of structural modifications in polysaccharide crystals during hydrothermal treatments in a pressurized cell

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1. Aim of the experiments

Polysaccharides such as cellulose and starch are the most abundant renewable resource and have a long history of utilization. Their structure have been studied since the early days of X-ray diffraction. However, the data were often limited by the crystalline quality of the samples. It was only recently that detailed 3D structures of cellulose, including hydrogen bond patterns, were determined using synchrotron X-ray and neutron diffraction of specimens containing aligned microcrystals. During processing, polysaccharides are often submitted to hydrothermal treatments that modify their structure or are used under high thermal stress. In addition, as they are polar molecules, water plays an major role in their structure and properties.

In 2005, we ran preliminary experiments consisting in heating cellulose, chitin and starch specimens in presence of water in pressurized helium *(french CRG D2AM beamline ; see proposal #02-01-670 and subsequent report)*. Modifications such as thermal expansion were successfully monitored using wide-angle X-ray scattering (WAXS) and phase transitions occurred at high temperature without any significant sample degradation. While those transitions have been known for years, very little data exist in literature on the structural aspects of such phenomena. With this new project, we wanted to follow the changes taking place in various model polysaccharide specimens by submitting them to specific pressure/heat cycles in presence of water, in order to elucidate the molecular details of the transitions.

2. Experimentals

Materials. Cellulose specimens consisted of native flax fibers, mercerized Ramie fibers and artificial fibers containing aligned tunicin or *Glaucocystis* microcrystals (**Figs. 1a,b**). Oriented specimens containing highly crystalline chitin were also prepared by cutting strips of material from the protective tubes of vestimentiferan sea worms. B. Shillito from Université Paris VI ("Adaptations aux Milieux Extrêmes" laboratory) kindly gave us dried tubes from *Lamellibracchia* and *Tevnia* worms (**Fig. 1c,d**) as well as never-dried material from *Riftia* worms collected at deep sea hydrothermal sources. Starch was used as a hydrated powder of highly crystalline spherulites prepared by recrystallization of synthetic amylose (**Fig. 1e**).

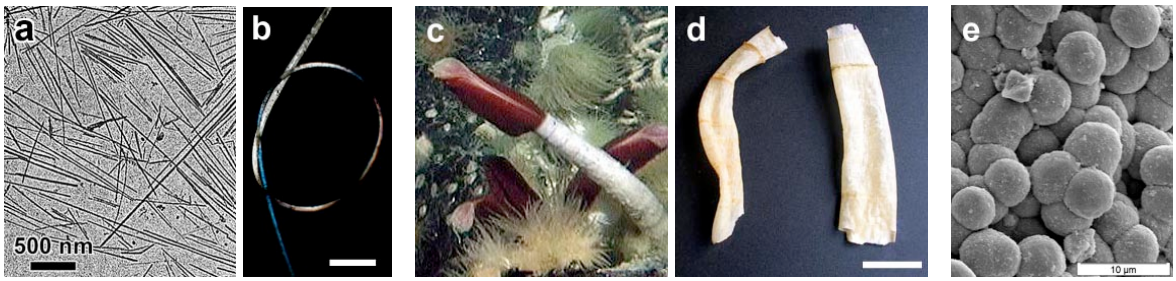

Figure 1. a) Tunicate cellulose whiskers (TEM image - bar : 100 nm) ; **b**) artificial fibers containing aligned cellulose whiskers (bar : 5 mm) ; **c**) *Tevnia* vent worm photographed close to a deep sea hydrothermal source ; **d**) dry tubes from *Tevnia* worms containing highly crystalline chitin microfibrils (bar: 2 cm) ; **e**) highly crystalline starch spherocrystals (SEM image).

Experimental set-up. A stainless steel specimen holder was designed for our experiments in order to hold fibrillar or powder samples in a glass capillary while having liquid in a surrounding container (**Fig. 2**). Cellulose and chitin, in the form of oriented fibers or strips of oriented material, were inserted into 1 mm o.d. glass capillaries (**Fig. 2a**). The sample were kept dry or soaked in water (**Fig. 2b**). Hydrated starch spherocrystals were poured into glass capillaries with (**Fig. 2c**) or without (**Fig. 2d**) liquid water. For all samples, depending on the experiments, the surrounding container was filled with water to prevent dehydration in the pressurized He environment **(Figs. b-d)**.

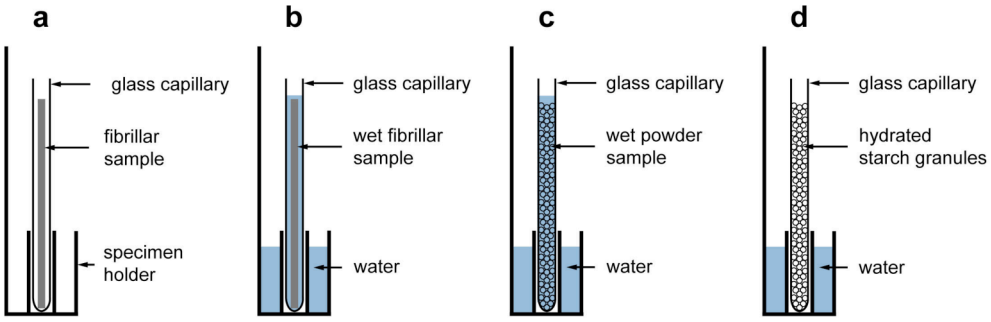

Figure 2. Sample set-up in the case of fibrillar (**a,b**) and powder specimens (**c,d**). The height of the specimen holder is about 2 cm.

The specimen holder was inserted in a sapphire tube (**Fig. 3a**) and placed inside the cell described in Testemale *et al.*, *Rev. Sci. Inst.* **76** (2005), 43905 (**Fig. 3b**). The chamber was pressurized using He, at pressures varying from 10 to 300 bars. The samples were then heated at a temperature varying from 25 to 300°C. The details about the heat-pressure treatments are given for each specimen in the Results section. The samples were probed by a X-ray beam at a 24 keV energy. WAXS patterns were recorded using a CCD detector during 5-60 s exposure times, with an angular range of ±11° defined by the exit window of the pressure cell (**Fig. 3c**).

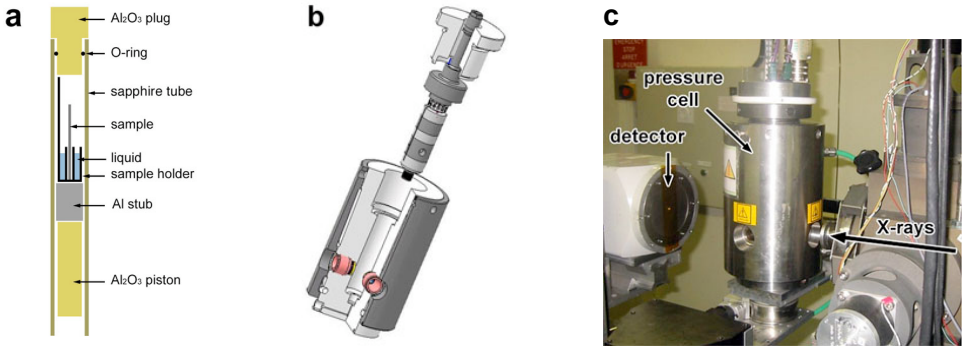

Figure 3. a) sample set-up in the sapphire cell ; **b**) scheme and **c**) picture of the pressure cell.

3. Phase transitions of polysaccharides in pressurized He

3.1. Starch

Native starch granules exhibit two kinds of X-ray powder diffraction patterns. The corresponding allomorphs, namely A and B, are described by a different packing of parallel double helices. When short amylose chains are recrystallized at high concentration, in presence of acetone or in pure water, sherulitic particles are formed that exhibit similar A and B-type patterns, respectively. A solid-state transformation from B to A is known to occur during a "heat-moisture" treatment used at the industrial scale to modify the functional properties of starch. A hydration of 35% is necessary for the transition to occur, at a temperature of 100-120°C. A solid-state transition from A to B has never been reported but when A-starch is solubilized in hot water, it recrystallizes into B-starch upon cooling.

In previous experiments, we have showed that native "potato amylopectin" starch granules could undergo a B→A transition in our pressure cell without any significant degradation *(see report #02-01-670)*. We thus carried a first set of experiments using similar conditions on B-type spherocrystals which possess a higher crystallinity. They were performed in He at 85 and 300 bars with liquid water in the external container.

As shown in **Fig. 3a**, the diffraction profile of allomorph B is clearly recognized up to 140°C. There is a slight shift to lower *q*-values due to thermal expansion. Contrary to what was observed on native starch granules, the general intensity did not significantly decrease at high temperature. From 150°C, the diffraction profile has clearly changed, now corresponding to allomorph A. As indicated by the total disappearance of the strong initial 100_B reflection, we estimated the transition temperature to be about 145°C. It is higher than the one measured in our previous experiments on native "potato amylopectin" starch (105°C). A comparison between the powder patterns recorded before (B) and after (A) the phase transition is shown in **Fig. 3b**. A similar heat-moisture treatment was performed at 300 bars, up to 160°C (5°C/min). The transition temperature was about 140°C which is roughly the same as that measured at 85 bars.

In these experiments, the transition was rather fast, occurring in a matter of minutes. This suggests that pressure has an influence on the heat-moisture treatment of starch. The fact that we successfully performed the B→A transition validates our experimental set-up. The initial 35% moisture that is necessary for the transition to occur did not significantly change throughout the pressure/heat treatment.

After peak indexing, we calculated the relative variation of lattice spacing corresponding to selected diffraction peaks with temperature. **Fig. 4** is a plot of such a variation for the 010_B, 110_B and 011_B peaks. The variation is generally linear although a more detailed analysis is required to detect possible different regimes. The determination of thermal coefficients and the study of thermal anisotropy from such data is under progress.

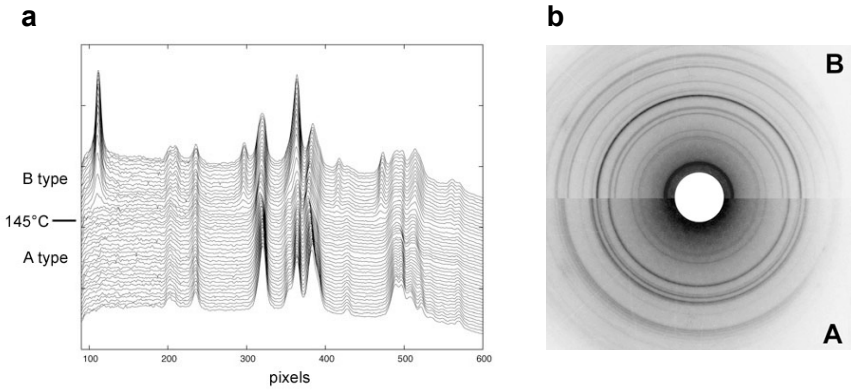

Figure 3. B→A phase transition in B-type spherocrystals (85 bars He). **a**) Diffraction profiles at different temperatures after subtraction of the diffusion background from He and sapphire cell. B-type and A-type profiles are recognized at 25°C in the initial *(top)* and final *(bottom)* spectra. **b**) Powder patterns of initial B-type *(top half)* and final A-type *(lower half)* at 25°C.

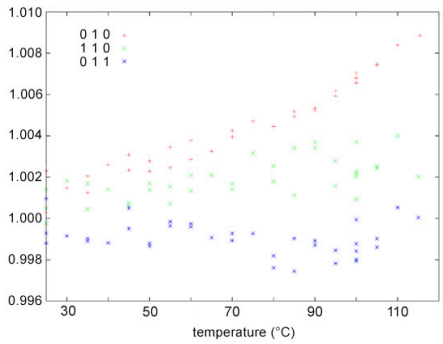

Figure 4. Relative variation of lattice spacing corresponding to selected diffraction peaks from B-type spherocrystals with temperature. The distances are normalized to the value obtained after linear extrapolation of the data to 0°C.

A second set of experiments was designed to perform B→A then A→B transitions on the same sample. In the first experiment, the B→A transition was first performed on hydrated spherocrystals (heating to 165°C at 5°C/min and cooling down to 25°C, at 20 bars). The cell

was then opened and water was injected in the capillary containing starch. The sample was pressurized and heated again until complete disappearance of the diffraction pattern (up to 185°C) when the spherocrystals melted/solubilized. It has to be noted that this temperature is significantly higher than the gelatinization temperature of native starch granules. This shows the very high thermal stability of the spherocrystals, certainly improved by the annealing effect of the first heating treatment. The sample was cooled down and, after 20 min at room temperature, a weak B-type pattern was observed, corresponding to the recrystallization of the sample.

A second experiment was performed with the spherocrystals in excess water, at 20 bars (**Fig. 5a**). Surprisingly, the B→A transition was detected at about 115°C, a temperature lower than that observed for hydrated powders. Melting/solubilization occurred at about 160°C and the sample was cooled down to room temperature. After a 20 min rest, a strong diffraction pattern was observed (**Fig. 5b**). Peaks of both allomorphs A and B were recognized. Up to 25 peaks were observed in this pattern, some of them with a width of only a few pixels.

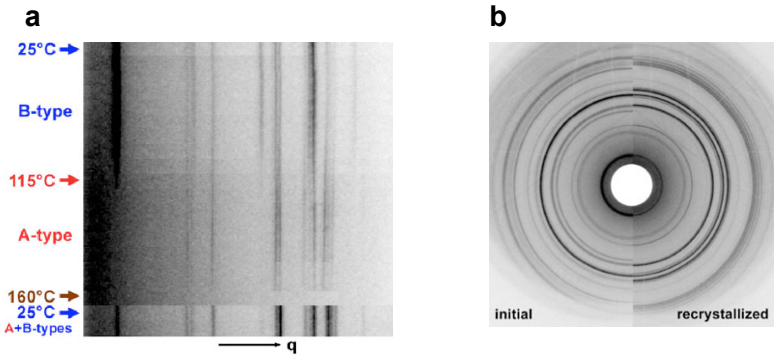

Figure 5. B→A phase transition in B-type spherocrystals in excess water (20 bars), followed by a melting and a recrystallization of the sample : **a)** variation of diffracted intensities with temperature ; **b)** comparison of the B pattern of the initial spherocrystals *(left)* with the final A+B pattern of the recrystallized sample *(right)*.

3.2. Chitin

Strips of a dry tube from a *Lamellibracchia* vent worm were cut, soaked in water and placed in a capillary. As the tube is known to have a plywood-like structure, the sample was oriented so that the beam was parallel to the chitin layers. As seen in **Fig. 6a**, highly oriented fiber patterns were recorded, corresponding to the β allomorph of chitin (with parallel chains). The samples were submitted to various heating/cooling cycles, up to temperatures of 330°C, at pressures of 100 and 300 bars. Two transitions were detected upon heating, at approximately 85 and 105°C, indicating the existence of different hydrated forms of β-chitin (**Fig. 6b**). When the sample was not heated above the second temperature, the transition were clearly reversible. When the second transition occurred, a hysteresis was observed upon cooling, with a unique transition at about 80°C. When the sample was heated up again, the transitions at 85 and 105°C took place again. The sharpness and high orientation of the fiber diffraction diagrams will allow to measure the thermal coefficients of β-chitin with precision, in presence of water or in the dry state. The effect of pressure on the transition will also be evaluated.

A similar hydrothermal treatment was performed on never-dried material from the protective tube of *Riftia* worms, soaked in water. In a temperature range of 25-40°C and at a pressure of 300 bars, we reproduced, to some extent, the environmental conditions of living deep sea worms. **Fig. 6c** is a diffraction pattern of β-chitin recorded in these conditions. The orientation in this sample was not as good as in *Lamellibracchia*, which may be explained by the fact the material was never-dried. However, the heat treatment yielded the expected transition temperatures : 85 and 110°C upon heating and 65°C upon cooling, with a clear hysteresis.

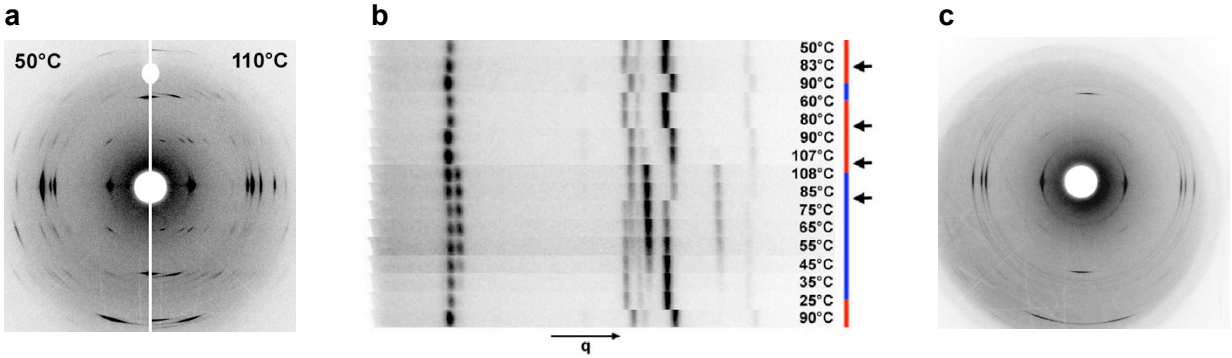

Figure 6. a) Two examples of diffraction diagrams recorded on hydrated strips of a tube from a *Lamellibracchia* vent worm, at 300 bars, and corresponding to two forms of β-chitin at 50°C *(left-half)* and 110°C *(right-half)*. **b)** Display of the equatorial reflections of β-chitin diffraction diagrams recorded during various thermal treatments of the same sample, at 300 bars. The red and blue vertical bands correspond to heating and cooling steps, respectively. The black arrows indicate phase transitions. **c)** Diffraction pattern of never-dried *Raftia* β-chitin in environmental conditions (20°C, 300 bars).

3.3. Cellulose

Native cellulose I contains two allomorphs with parallel chains, namely I_α and I_β. We heated dry and wet fibers of aligned *Glaucocystis* I_α microcrystals up to 280°C, at 100 bars. Up to 260˚C, the thermal expansion was reversible and cellulose I_α exhibited a rather constant expansion coefficient, perpendicularly to the glocopyranose units (**Fig. 7**). Then, at 260°C, a fast and irreversible crystal transition occurred. Upon cooling, a transition was observed at about 220°C but to another form of I_β.

The 002 reflection corresponds to the length of one anhydrous glucose. Its intensity is weak in native cellulose because the neighboring chains are translated with respect to each other by half a glucose unit along the chain direction. At 260°C, the 002 intensity became much stronger, indicating a longitudinal shift of the molecules.

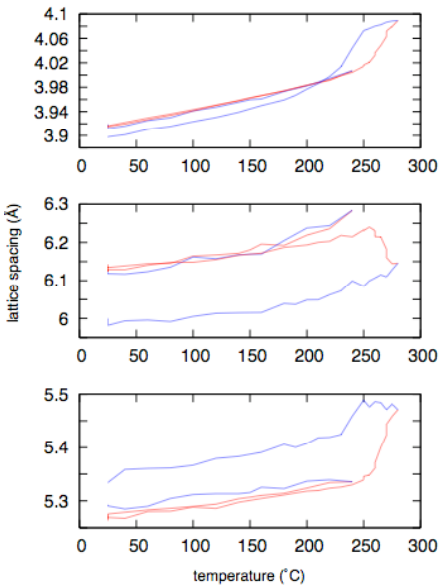

Figure 7. Evolution of lattice spacing of the three measure spots. The 3.9Å spacing corresponds to the distance between the pyranose plane. Red lines indicate the heating process and the blue line, the cooling process.

4. Conclusion and perspectives

The thermal properties of dry, hydrated or wet highly crystalline polysaccharide specimens have been successfully monitored *in situ* by combining heat-pressure treatments and synchrotron WAXS analysis. Preliminary but substantial knowledge on the molecular mechanisms involved in a number of structural transitions have been obtained. A very large amount of data has been recorded during our experiments and their treatment is still in progress.

It would be important to record data at higher resolution but the design of a new pressure cell with a larger collection angle is required. In addition, it would be interesting to cool down the specimens in order to study their thermal properties at temperatures lower than 20°C. In this case too, the pressure cell and temperature control system would have to be redesigned.