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What Factors Determine Hierarchical Structure of Microbial Cellulose – Interplay among Physics, Chemistry and Biology –

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Summary: A microbial cellulose film (pellicle), prepared by *Acetorbactor xylinum,* is a supramolecule system, absorbing a huge amount of water (99% by weight). To elucidate the affinity to water, we investigated the hierarchical structure in a pellicle by using ultra-small-angle and small-angle neutron scattering, observing a wide range of length scales from nm to μm. We successfully determined mass fractal dimensions for the amorphous structure, which hierarchically varies local concentration fluctuations, bundle and network, appeared as the length scale increases. On a basis of these findings, we discuss to address a question what factors determine the hierarchy in the microbial cellulose. This is a new topic of soft matter science, regarded as *reaction-induced self-assembly* in a non-equilibrium open system, therein soft matter physics, biochemistry and cell biology crucially interplay.

Keywords: cellulose; neutron scattering; reaction-induced self-assembly

Introduction

Cellulose is the most abundant biopolymer on earth, synthesized by a wide variety of living organisms as well as by higher plants.[1,2] It is a linear homo polysaccharide, composed of β-1,4-linked glucans with a high level of symmetry in molecular structure. The glucan unit is amphiphilic because its surface and side are hydrophobic and hydrophilic, respectively. The intrachain hydrogen bonding makes a cellulose chain stiff and therefore crystalline. The crystallite (or microfibril) in the cellulose texture excludes water molecules and is crucial for physicochemical stability. The affinity to water, on the other hand, is tightly related to amorphous domains in the cellulose texture, where ''amorphous'' means a space occupied by the cellulose swollen by water or by pure water.

In this paper, we discuss the hierarchical amorphous structure in ***"pellicle"*** (microbial cellulose film), produced by bacteria genera *Acetobactor Xylinum*.[3] The pellicle is a supramolecular system, surprisingly containing water up to 99% by weight (see Figure 1). The formation of hierarchical structures in the pellicle involves a nearly simultaneous and subsequent process of polymerization, crystallization and molecular assembly. The hierarchical structure in the microbial cellulose is determined not only by the molecular structure of cellulose of β-1,4-linked amphiphilic glucans in addition to physicochemical factors, imposed under a cultivation process (temperature, agitation conditions etc.), but also by the biogenic factors originating from the prokaryotic *and* aerobic genera of *A. xylinum*, Especially, terminal complexes (TCs), reaction sites allocated on the outer membrane, and their regulation are crucial (Figure 2). This is a new topic of soft matter science as related to a non-equilibrium

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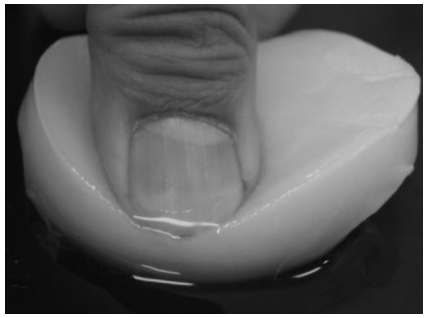

Figure 1.
Photo of a pellicle swollen by a huge amount of water (99% by weight). The color of pellicle is significantly turbid, suggesting strong heterogeneity in it. By compressing, the pellicle easily releases water to outside.

open system, therein physics, chemistry and biology interplay.

Our key method to elucidate the hierarchical structure is an ultra-small-angle and small-angle neutron scattering technique (SANS & USANS). The combination of USANS and SANS is advantageous for in-situ observation over for a wide length scales from nm to 10 μm. If we simply replace water with deuterated one (D_2O), without drying or staining, neutron scattering visualizes spatial distribution of water in amorphous domains of a microbial cellulose gel, which is a counterpart of the center of mass for crystallite. As a results of SANS and USANS measurements, we quantitatively examined

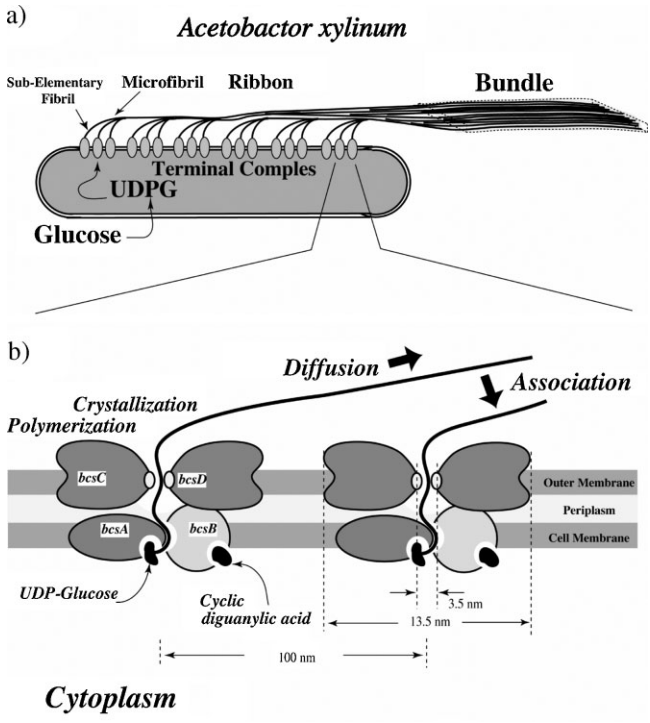

Figure 2.
(a) Schematic diagram of a single bacterium, *Acetorbactor xylinum*, which is gram negative and prokaryotic. A single cell possecesses a number of cellulose synthesizing sites, called "*terminal complex*". synthesizing elementary fibrils by using glucose and/or UDP-glucose. Form a terminal complex (TC), those are arranged in a linear row on the outer membrane. A sub-elementary fibril composed of several cellulose chains is extruded from the pore of TC and assembled into a microfibril, ribbon and bundle. (b) Schematic diagram, highlighting two TCs, each of which is composed of four proteins, A B C and D. Protein A (cellulose synthetase), on the inner membrane facing to cytoplasma, recognizes UDP-glucose and synthesizes it into cellulose. Proteins C and D, on the outer membrane are supposed responsible for crytallization and extrusion of cellulose chains. Protein B regulates cellulose synthesis by binding cyclic diguanylic acid.

self-assembled structure of networks, bundles and local concentration fluctuations inside the cellulose bundle, which are hierarchically organized by as the length scale decreases from micro meter to nanometer. A key parameter we obtained is mass fractal dimension (d_m) describing the amorphous structure. Based on these experimental findings, we discuss what factors determine the hierarchical structure of microbial cellulose, therein the interplay among physics, chemistry and biology is crucial.

Experimental Part

We employed two USANS spectrometers at research reactor JRR-3, at Japan Atomic Energy Agency, Tokai, Japan. A focusing USANS spectrometer (SANS-J-II)[4] was newly constructed using focusing compound lens (MgF_2) to cover a q-region of USANS ($10^{-4} < q < 0.003\,\text{Å}^{-1}$),[2] where q is defined as $q = 4\pi/\lambda sin(\theta)$ with scattering angle 2θ and wave length λ. SANS-J-II is able to choose conventional pinhole SANS collimations without using focusing lenses, in order to cover $0.003 < q < 0.1\,\text{Å}^{-1}$. To reach to the USANS q-region of $10^{-5}\,\text{Å}^{-1}$ order, we utilized a double crystal (Bonse-Hart) USANS spectrometer (PNO) at JRR3, Tokai. By using grooved perfect silicon crystals and thermal neutron of $\lambda = 2\,\text{Å}$, we can cover from 3×10^{-5} to $10^{-4}\,\text{Å}^{-1}$.

Preparation of Microbial Cellulose

A. xylinum, coded as ATCC strain 53582 (NQ-5), was cultivated in a petri dish, containing of 200 cc of Hesrin-Schramm (HS) culture medium[5] (D-Glucose 2.0%, bacto peptone 0.5%, bacto yeast extract 0.5%, citric acid 0.115%, disodium hydorgenphosphate 0.27% by weight and Ph = 6.0). After incubation of 2 weeks at 30 °C, we obtained a pellicle of 1 cm thickness, floating at the top of the culture medium. To obtain pure cellulose without containing bacteria, we cut the pellicle at the air side into a film specimen of 2∼5 mm thickness. For large scattering contrasts, the film specimen of cellulose, cultivated in the HS culture medium in H_2O, was first immersed in a large amount of D_2O over several nights and H_2O was replaced with D_2O.

Experimental Results

We studied the swollen and dried cellulose by using SANS and USANS. Figure 3

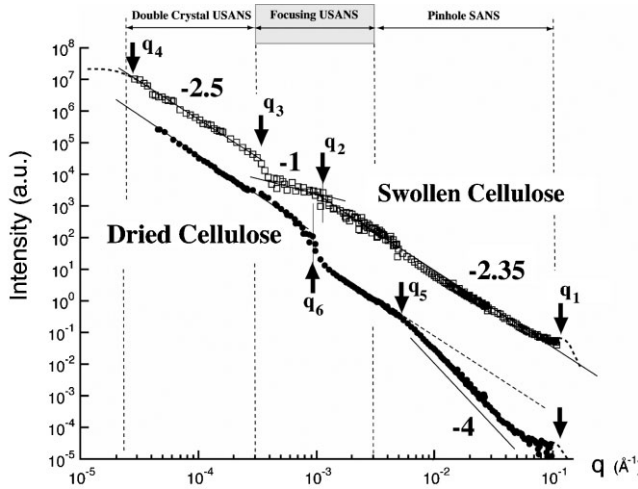

Figure 3.
Scattering q-profiles obtained for swollen and dried cellulose. A whole q-range from 3×10^{-5} to 0.1 Å^{-1} is covered by combining pinhole SANS, focusing USANS and double crystal USANS.

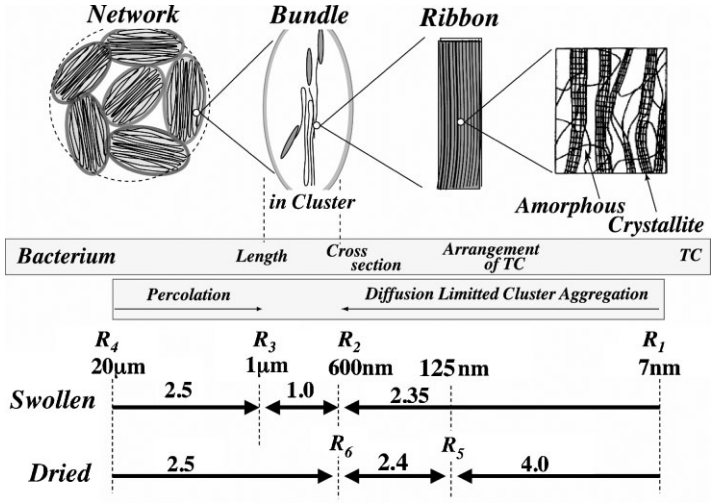

Figure 4.
Mass fractal dimensions (d_m), determined by neutron scattering are summarized for swollen and dried cellulose. d_m changes as related to network, bundle and local concentration fluctuations.

exhibits our results, (or shown as Figure 4 in ref. [6]). For the swollen cellulose, the scattering curve is characteristic to exhibit a power law q-behavior ($\sim q^{-\alpha}$). From higher q values, we found $\alpha = 2.35$ for $0.001 < q < 0.07\ \text{Å}^{-1}$, $\alpha = 1$ for $0.0003 < q < 0.001\ \text{Å}^{-1}$, and $\alpha = 2.5$ for $0.00003 < q < 0.0003\ \text{Å}^{-1}$, respectively. The upper and lower limits for the power laws are denoted as q_1, q_2, q_3 and q_4, respectively. At q_1 or q_3, there is a hamp or drop, respectively, deviating from the power laws. Figure 4 summarizes the power laws and length scale limits determined by scattering. A model function, for example proposed by Beaucage,[7] is used in order to quantitatively analyze our SANS&USANS and to obtain structural parameters, which will be our a future work.

We attribute $\alpha = 1$ found in the middle q-region ($q_3 < q < q_2$) to a bundle, composed of the cellulose ribbons swollen by water. q_2, upper q-limit for $\alpha = 1$, indicates a cross sectional diameter of the bundle, evaluated as $R_2 = 600\,\text{nm}$ ($=2\pi/q_2$). $\alpha = 2.5$ in the lowest q region is supposed due to a gel network, composed of the bundles. $\alpha = 2.35$ in the highest q-region is due to concentration fluctuations inside the swollen cellulose bundle. The cellulose swollen by D_2O or pure D_2O has a large scattering contrast to the crystallite. The power law of $\alpha = 2.35$ is limited by a hamp appearing at q_1 ($=0.07\ \text{Å}^{-1}$), The size R_1 ($=2\pi/q_1$) is about 7nm, which is equal to the crystallite width in the cellulose ribbon, as reported in ref. [3].

For the dried cellulose, the power law q-behaviors were strongly affected. We found $\alpha = 4$ for higher q values of $0.005 < q < 0.05\ \text{Å}^{-1}$, $\alpha = 2.4$ for $0.001 < q < 0.005\ \text{Å}^{-1}$, and $\alpha = 2.5$ for $0.00003 < q < 0.0008\ \text{Å}^{-1}$. We denoted the upper and lower limits for the power laws as q_5 and q_6, in Figure 3.

In the highest q-region, we clearly observe a Porod's law, showing an asymptotic decay of q^{-4}, originating from a sharp interface in the dried cellulose. The space occupied by water might become vacant after drying. The upper q-limit for $\alpha = 4$ is close to q_1, and the hump at q_1 becomes more obvious as compared to the swollen cellulose. From the lower q-limit for $\alpha = 4$ (q_5), we evaluated $R_5 = 125\,\text{nm}$ ($= 2\pi/q_5$), which agrees well with the ribbon width reported in ref. 3. In the swollen pellicle, the diameter of bundle was determined as $R_2 = 600\,\text{nm}$, may be composed of several ribbons, the width of which was determined as $R = 125\,\text{nm}$ after drying.

Thus the internal structure (or internal concentration fluctuations) of the cellulose bundle is strongly affected by drying. After drying, the q-region showing $\alpha = 2.5$ from the gel network increases from q_3 to q_6. This is due to the shrinkage of the gel network. If we compare two q values (q_3 and q_6), we obtain a swelling ratio about 2.7.

Discussion

Biochemistry and Cell Biology

Biosyntheic Pathway

First we summarize a biosyntheic pathway.[1] *A. xylinum*, prokaryotic and gram negative bacterium, synthesizes an extracellular fibril of cellulose using glucose, as follows,

$$\mathrm{UDP-glucose} + [\beta\text{-}1,4\text{-glucose}]_n \rightarrow \mathrm{UDP} + [\beta\text{-}1,4\text{-glucose}]_{n+1}.$$

A single cell polymerizes up to 200,000 glucose residues per second into β-1,4-linked glucan chains. The chemical reaction, denoted above, is composed of four enzymatic steps, completing a whole pathway from glucose to cellulose, which have been characterized in cell-free extracts of *A. xylinum*. The four steps are given as follows,

- the phosphorylation of glucose by the enzyme glucokinase,
- the isomerization of glucose -6-phosphate to glucose-1-phosphate by the enzyme phosphoglucomutase,
- the synthsis of UDP-glucose by the enzyme UDPG-pyrophosphorylase,
- the enzyme cellulose synthetase reaction using UDP-glucose as a substrate.

Based on the four steps in a pathway, the total cost, incurred in incorporating one glucose residue into polyglucan, is only two of phosphate bond energy. Step (iv) (cellulose synthesis) takes place on a cytoplasmatic membrane. It is highly catalyzed at a complex of proteins called as ''terminal complex'', containing the enzyme cellulose synthetase.

Terminal Complex and its Regulation

Next we summarize key functions of *A. xylinum* related to cell biology. Cellulose synthesis (step (iv)) occurs in association with TCs in a plasma membrane of the cell.[1] Figure 2 (b) schematically shows two TCs, based on ref 8 and 9. TC is a complex of proteins, composed of a number of proteins, coded by genes in the cellulose-synthesizing operon. The protein (*bcsA*) in an inner membrane is the cellulose synthetase, responsible for recognition and synthesis of UDP-glucose (reaction (iv), as denoted above). Then crystallizing and extrusion of cellulose fibers ($[\beta$-1,4-glucose$]_{n+1}$) might be controlled by the proteins (*bcsC* and *bcsD*) in an outer membrane. The protein (*bcsB*) is allocated in an inner membrane and interacts with a novel nucleotide, cyclic diguanylic acid (c-di-GMP) to regulate the cellulose synthetase (*bcsA*).[10] TC has a 6-fold structure of protein complexes, which allows to polymerize several chains simultaneously. The sizes of TC and its pore are about 13.5 and 3.5 nm, respectively. TCs (number of TC is about 100 for *A. xylinum*) are allocated in a row on the outer membrane (linear allocation). A mean distance between TCs is about 100 nm, (Figure 2 in ref. [11]).

Cell Division

Peptone and yeast in the HS medium contain nitrogen which is necessary for gene duplication and therefore cell division. A typical turnover time of cell division is reported about 20 min. Thus in the culture medium spending about 2 weeks, cell division occurs simultaneously as well as cellulose synthesis. We expect that cell division causes a branch of cellulose bundle, as discussed later in Figure 5.

Cell Movement

The synthesis of linear polysaccharide chain can be involved in generating movement of entire cells.[12] Cellulose synthesis in the bacterial cell gives rise to a rotational

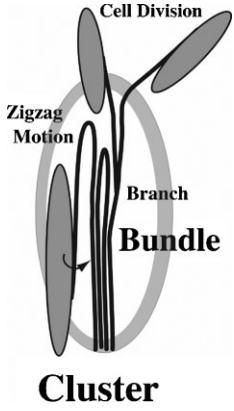

Figure 5.
Schematic view of a *cluster*, in which bacteria (yellow) coherently move and cellulose bundle (black) is formed. A bundle becomes by "zigzag motion" and cell division makes a bundle branched.

movement of the cells along their longitudinal axes as they extrude and spin the cellulose fibers. Bacteria move forward, when they extrude cellulose backward. Due to aerobic nature of *A. xylinum*, it tends to move toward the aerobic circumstances. *A. xylinum* lives near a liquid surface of the medium and as the pellicle becomes thicker, it moves toward deeper.

Physics -Fractal Analysis

We found that small-angle scattering obeys a power law q-behavior according to $q^{-\alpha}$ as a function of a magnitude of scattering vector q and that α varies between 1 and 3. We attribute α to mass fractal[13] due to distribution of the center of mass for crystallite (microfibril) in amorphous cellulose swollen by water. α is identical mass fractal dimension d_m.

To discuss the hierarchical structure of microbial cellulose, we introduce a key domain of ***cluster*** (Figure 5), in which bacteria coherently move due to chain constraint and cellulose chains are aligned parallel. As denoted, during cultivation in medium, *A. xylinum* simultaneously behaves cellulose synthesis, cell division and cell movement. For the length scale of a cluster, we determined α (or d_m) = 1, attributed to a bundle in a cluster.

Inside a cluster of swollen cellulose, α (or d_m) = 2.35, we determined, is interpreted as follows. For a direction parallel to the bundle (z-axis), the dimension should be $d_m = 1$. If we postulate that the mass is equally distributed in a xy plane, we obtain $d_{my} = d_{mz} = 0.67$ (=(2.35−1)/2).

A model of diffusion limited cluster aggregation (DLCA)[12] considers a spherical primary particle and predicts that $d_m = 1.75$. In this model, the mass is distributed equally in x, y and z directions therefere, we obtain $d_{mx} = d_{yx} = d_{mz} = 0.58$ (=1.75/3). We now recognize discrepancy between swollen cellulose ($d_{mi} = 0.67$) and the DLCA model ($d_{mi} = 0.58$), where a subscript "i" denotes x or y. As described in Figure 2 (b), the cellulose chains, which are extruded from adjcent TCs, diffuse and are associated side by side. A rigid cellulose chain is characteristic for "***an-isotropic excluded volume interaction***", which influences on an association process. It should be denoted that d_m is found to be larger, in case of the systems with an isotropic excluded volume interaction, such as actin bundle formation[14] and colloidal rod aggregation (boehmite).[15] For the network beyond a cluster, we determined $d_m = 2.5$ by using USANS. A percolation (network) model[12] predicts $d_m = 2.53$ for a three-dimensional system, which agrees with our experimental findings.

On a basis of mass fractal dimension d_m and upper and lower length scale limits (R_L and R_U), we evaluate a volume fraction of cellulose ϕ(R), according to $\phi(R) = [R_U/R_L]^{-(3-dm)}$. For the swollen cellulose, we employ $R_L = 7$ nm (size of microfibril (crystallite)), $R_U = 600$ nm (size of bundle) and $d_m = 2.35$. Surprisingly, we find that a large amount of water about 90% is involved in a bundle structure.[6] The network structure, on the other hand, involves only 10% of total water. The bulk concentration 1 wt% appears for the length scale of 20 μm. Beyond 20 mm, ϕ(R) does not vary depending on the length scale of observation R.

If we compress the pellicle by a finger, it easily releases water to outside (Figure 1).

A trick of high water content for the pellicle is ''***capillarity***'', therein water molecules **"*impregnate*"** or **"*intercalate*"** into hierarchical amorphous domains in the pellicle.[16] Capillarity is generally proportional to $\Delta\gamma/R$, where $\Delta\gamma$ is difference in surface tension between wetting and de-wetting states.[17] The speciality for pellicle is that an amorphous domain size R hierarchically varies according to a mass fractal manner in length scales from nonometers to miron meters, as determined by SANS and USANS. To the contrary, a hydrogel, such as poly (N-isopropylacrylamide) gel, tightly binds water molecules and hardly releases water by compression.[16] This is due to strong hydration (hydrogen bonds formation) around a hydrophilic amide group.

Artificial Cellulose Synthesis

Although the in-vitro synthesis of cellulose has been the most challenging research subjects, the first successful achievement via. enzymatic reaction was reported in 1991.[18] The reaction is designed to use the substrate of β-cellobiosyl fluoride, dissolved in the acetate buffer (CH_3COOH/CH_3COONa). To this solution, acetonitrile is added. The polymerization is initiated by mixing this substrate with enzyme solution, unpurified crude cellulase obtained from *Trichoderma viride*. With this method, cellulose is successfully synthesized. However, the degree of polymerization is rather small about $N = 20$.

The hierarchical structure, observed in the cellulose synthesized by a non-biosynthesis pathway (in-vitro ''enzymatic polymerization'' of cellulose), is completely different from the microbial cellulose. We employed this enzymatic polymerization method to investigate the molecular assembly *in-vitro*.[19] The solution was transparent up to 2 h after starting the polymerization, however, becomes opaque and turbid. As the polymerization was further proceeded, white precipitates of the reaction product (polysaccharide) appeared in the solution, indicating the self-assembly of cellulose, synthesized by in-vitro enzymatic polymerization, is water-insoluble. The precipitate was examined over wide q region by using SANS & USANS, partially reinforced by small-angle or ultra-small-angle X-ray scattering (SAXS and USAXS) (see Figure 10 in ref. [19]). The scattering curve is described by the power law of $\alpha = 3.7$ in the lower q, and $\alpha = 2.1$ in the middle q and finally $\alpha = 4$ at the highest q. $\alpha = 3.7$ and 4 found in the lowest and highest q are attributed to surface structure of the precipitate, according to surface fractal, the dimension of which is defined as $d_S = 6\text{-}\alpha$. In the lowest q, surface fractal dimension of the precipitate was determined as $d_s = 2.3$ (=6–3.7), implying the sharp surface of crystallite. Appearance of surface fractal, instead of mass fractal dominantly observed for microbial cellulose, means that the precipitate is homogeneous excluding water.

For the artificial synthesis, using the unpurified crude cellulase, it is found that appears the stable crystalline polymorph of Cellulose II, in which the cellulose chains are anti-parallel to each other. On the other hand, Cellulose I is obtained for the purified cellulase.[20] These findings indicate that a mean distance between two active synthesizing sites seems important; if this distance is long enough, a cellulose chain extruded from an active site is allowed to rotate 180° with respect to its neighbouring residue and to form anti parallel packing of Cellulose II. A mean distance between two active sites influences on physical processes in an outer cell matrix of diffusion, rotation and then aggregation.

What Factors Determines Hierarchical Structure of Microbial Cellulose?

Acetobactor xylinum is a micro-machine for cellulose biosynthesis, with elegant nano-machines of TCs arranged in a linear row on the outer membrane. As indicated in Figure 3, the hierarchical structure found in the pellicle varies three different ways, i.e., a network, bundle and local fluctuations. The intermediate structure, bundle in a cluster, appears in a length scale as large as the bacterium body, the length and cross

section of which are order of 1.0 μm and 0.5 μm.

Hierarchy inside a Cluster

A *sub-elementary fibril*, composed of several (or 16) elementary fibrils of β-1,4-linked glucans, is extruded from one TC. The cross section of *sub-elementary fibril*, which is reported about 1.5 nm,[3] might be affected by a pore size of TC (~3.5 nm). In a sub-elementary fibril, we find a meta-stable crystalline polymorph of Cellulose I, which means in a TC, the polarity of cellulose chains is controlled identical among all chains. A sub-elementary fibril diffuses and aggregates side by side with those extruded from the neighboring TCs. Then appears a *microfibril*, which seems a crystalline domain (a crystallite) minimum in size. SANS showing the upper q-limit of $q^{-2.35}$ determined the cross section as R_1 (~7 nm). For the length scales beyond R_1, *microfibril*s are expected to further associate into a *cellulose ribbon*. According to our SANS measurements, we observed the power law scattering of $q^{-2.35}$, which is attributed to local concentrations is the swollen cellulose.

Agents added to the culture medium, such as direct dyes, fluorescent brightening agents, and cellulose derivatives, attach to the cellulose chains and prevent self-assembling process of the microbial cellulose [chapter 5 in ref. 3]. Direct dyes, and fluorescent brightening agents are small enough to separate into sub-elementary fibrils, whereas cellulose derivatives are larger to cause the subunit about 10 nm, which is regarded as a microfibril.

A few TCs in a linear row are grouped. The sub-elementary fibrils from the same group of TCs might be extruded coherently and give rise to crystalline order of a microfibril. An unsettled problem about cellulose biosynthesis is the regulation of cellulose synthesis and *coherency in turnover in one TC, or among TC's*, which must strongly affect fibril formation and crystallinity in it.

The tablet shape of cellulose ribbon might be related to the linear allocation of TC. However, when we observed the swollen pellicle by SANS, it should be addressed again that the ribbon is not obvious. The power law scattering $q^{-2.35}$, due to the amorphous structure, appears continuously crossing the size of cellulose ribbon on about 100 *nm*. In the case of dried cellulose, on the other hand, we recognized it as a q position (q_6), where a Porod law of q^{-4} starts to appear.

Hierarchy beyond a Cluster

The microbial cellulose finally accumulates into a pellicle that floats on top of the culture medium and entraps *A. xylinum.* In a length scale region beyond the length of *A. xylinum* (~μm), cell movement and cell division are also important to determine the hierarchy of the microbial cellulose. The network structure of $\alpha = 2.5$ was obtained by a static cultivation at the air surface of culture medium, in which *A. xylinum* is able to freely move. *Percolation of clusters* might occur in a plane parallel to the air surface.

If we use a template to control cell movement, we are able to modify the network structure and its fractal dimension. For example, a honeycomb patterned cellulose network was fabricated by controlling the cell motion of *A. xylinum* on a patterned template.[21] The highly orientated cellulose is also obtained by employing a grooved substrate of polydimethylsiloxane (PDMS).[22,23] Due to excellent permeability of oxygen of PDMS, *A. xylinum* tends to attach at PDMS surface and move along the groove, when we put the PDMS substrate at interface between air and culture medium. Similarly, an external field such as agitation might influence on the network structure beyond μm. This is applied to create the molded cellulose objects.[24]

Conclusions

We discussed the microbial cellulose, in which the amorphous structure is hierarchically organized according to a mechanism of *reaction-induced self-assembly,* occurring

around a living cell. SANS & USANS, covering over a wide range of length scales from nm to 10 μm, determined mass fractal dimensions, related to local concentration fluctuations, bundle and network, those appear as length scale increases. In an open and non-equilibrium system of living cell, the interplay among physics, chemistry and biology plays a crucial role to determine a hierarchical structure of the pellicle.

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