Enzymatic synthesis of cellulose in space: gravity is a crucial factor for building cellulose II gel structure

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Abstract

We previously reported *in vitro* synthesis of highly ordered crystalline cellulose II by reverse reaction of cellodextrin phosphorylase from the cellulolytic bacterium *Clostridium* (*Hungateclostridium*) *thermocellum* (CtCDP), but the formation mechanism of the cellulose crystals and highly ordered structure has long been unclear. Considering the specific density of cellulose versus water, the formation of crystalline and highly ordered structure in an aqueous solution should be affected by gravity. Thus, we synthesized cellulose with CtCDP at the International Space Station, where sedimentation and convection due to gravity are negligible. Optical microscopic observation suggested that cellulose in space has a gel-like appearance without apparent aggregation, in contrast to cellulose synthesized on the ground. Small-angle X-ray scattering (SAXS) and wide-angle X-ray scattering (WAXS) indicated that cellulose synthesized in space has a more uniform particle distribution in the ~100 nm scale region than cellulose synthesized on the ground. Scanning electron microscopy (SEM) showed that both celluloses have a micrometer scale network structure, whereas a fine fiber network was constructed only under microgravity. These results indicate that gravity plays a role in cellulose II crystal sedimentation and the building of network structure, and synthesis in space could play a role in the design of unique materials.

Keywords: *cellulose, cellodextrin phosphorylase, synthesis in vitro, microgravity*

Declarations

Funding: This work was supported by JSPS KAKENHI Grant Number 19K15884 (grant to NS), by a Grant-in-Aid for Innovative Areas from the Japanese Ministry of Education, Culture, Sports, and Technology (MEXT; grant no. 18H05494 to KI), and the Academy of Finland through research grant SA-FOSSOK [Decision

No. 309384]. K.I. thanks the Finnish Funding Agency for Innovation for the support of the Finland Distinguished Professor Program “Advanced approaches for enzymatic biomass utilization and modification (BioAD)”.

Conflicts of interest/Competing interests: Not applicable.

Code availability: Not applicable

Ethics approval: Not applicable

Consent to participate: Not applicable

Consent for publication: Not applicable

Acknowledgments

The authors thank Prof. Motomitsu Kitaoka of Niigata University for providing *CtCDP* gene and Prof. Tomoya Imai of Kyoto University for helpful discussions. The WAXS and SAXS experiments were conducted at the BL8S3 station of Aichi Synchrotron Radiation Center, Aichi Science & Technology Foundation, Aichi, Japan (Proposal No. 2020D6031). SEM observation was conducted under the supervision of Dr. Satoshi Kimura of the University of Tokyo. We thank Confocal Sciences Inc. and Japan Manned Space Systems Corporation (JAMSS) for sample preparation and launch to the ISS.

Graphical Abstract

60 **Introduction**

61 Cellulose is the most abundant carbohydrate on Earth, and has been utilized by
62 humans from ancient times. In nature, cellulose is mostly produced by woody and
63 herbaceous plants as a cell-wall component. It is also synthesized by some
64 microorganisms such as *Komagataeibacter xylinus* (*Acetobacter xylinum*),
65 invertebrate animals (urochordates), or green algae (*Cladophora* species)
66 (VanderHart and Atalla 1984; Belton et al. 1989; Larsson et al. 1997). Cellulose is
67 a linear polymer of exclusively β -1,4-glycosidic-bonded glucose molecules
68 synthesized by cellulose synthase complex on the cell membrane of these species.
69 β -1,4-Glucan chains synthesized by the complex on the cell membrane
70 spontaneously assemble and crystallize to form cellulose microfibrils (CMF; also
71 called cellulose nanofibers, CNF). The shape of CMF depends on the geometry
72 and morphology of the cellulose synthase complex (Brown 1996; Saxena and
73 Brown 2005), but the mechanism of CMF formation is still unknown. Inside
74 CMF, the β -1,4-glucan chains are bound together by hydrogen bonds and
75 hydrophobic interaction to form a specific crystalline structure. Cellulose I_α and I_β
76 are the smallest crystalline units of natural cellulose, and these two natural
77 crystalline allomorphs are composed of glucan chains in parallel orientation
78 (Atalla and VanderHart 1984; Nishiyama et al. 2002, 2003). In contrast, cellulose
79 II is a non-natural crystalline form originally found in mercerized and regenerated
80 cellulose. The crystalline structure of cellulose II is significantly different from
81 those of natural cellulose I_α and I_β , having an anti-parallel orientation of cellulose
82 molecules (Kolpak and Blackwell 1976; Langan et al. 1999; Kim et al. 2006).
83 Cellulose II may be thermodynamically more stable, considering that it is formed
84 in preference to metastable cellulose I_α or I_β when dissolved β -1,4-glucan chains
85 are recrystallized.

86 To elucidate the formation mechanism of CMF and to develop new
87 materials applications, synthesis of various forms of artificial cellulose has been
88 attempted (Uryu et al. 1983, 1985; Nakatsubo et al. 1996). Early efforts showed
89 poor regio- and stereo-selectivity, and thus highly substrate-selective enzymatic
90 approaches were adopted (Kobayashi et al. 1991, 2000; Kobayashi and Shoda
91 1995; Kobayashi 2005; Tanaka et al. 2007). Cellodextrin phosphorylase (CDP) is
92 one of the enzymes utilized for the synthesis of cellulose *in vitro*. Although CDP
93 catalyzes phosphorolysis of cellodextrin (cellooligosaccharide), it is possible to

94 synthesize cellulose via the reverse reaction by using high concentrations of α -D-
95 glucose-1-phosphate (α -G1P) as a glycosyl donor, with glucose and cellobiose as
96 primary glycosyl acceptors (Alexander 1968; Sheth and Alexander 1969;
97 Krishnareddy et al. 2002). The glycosyl donors form β -1,4-glycoside bonds with
98 the non-reducing ends of glycosyl acceptors. In this manner, platelet lamellae of
99 crystalline cellulose having the degree of polymerization (DP) 9 were formed *in*
100 *vitro* (Hiraishi et al. 2009). All these studies aiming to synthesize cellulose *in vitro*
101 afforded cellulose II. Pylkkänen *et al.* have found that concentrated cellulose II
102 synthesized by CDP from *Clostridium (Hungateiclostridium) thermocellum*
103 (CtCDP) formed crystalline platelet lamellae and ribbon-like higher-ordered
104 network structure (Pylkkänen et al. 2020). However, the mechanism of
105 formation of cellulose II's supermolecular structure is still unknown, as is that of
106 natural cellulose I $_{\alpha}$ and I $_{\beta}$.

107 Protein crystallization in space enhances the quality of protein crystals due
108 to decreased sedimentation and convection under microgravity (Vekilov 1999).
109 This affords more orderly crystals than can be obtained on the ground, enabling
110 researchers to obtain higher-quality X-ray diffraction data (Snell et al. 1995; Inaka
111 et al. 2011; Nakamura et al. 2015; Tachioka et al. 2017; Yamaguchi et al. 2021).
112 A crystal of alloy semiconductor grown on the International Space Station (ISS)
113 also showed better quality than one grown on the ground (Inatomi et al. 2015),
114 and an NaCl crystal grown on the ISS had different morphology from a crystal
115 grown on Earth (Fontana et al. 2011). On the other hand, the synthesis and crystal
116 formation of organic polymers such as cellulose under microgravity in space have
117 not yet been investigated.

118 In the present study, cellulose II was synthesized *in vitro* using CtCDP on
119 the ISS. We investigated how gravity affects cellulose II crystalline or higher-
120 order structure formation by comparing the product with material synthesized in
121 the same way on the ground, employing small-angle x-ray scattering (SAXS),
122 wide-angle X-ray scattering (WAXS), and scanning electron microscopy (SEM).

123

124 **Materials and Methods**

125 **Materials.**

126 α -G1P and pET-28b vector were purchased from Sigma-Aldrich Co. LLC (MO,
127 US). Cellobiose and other chemical reagents were purchased from FUJIFILM
128 Wako Pure Chemical Corporation (Osaka, Japan). Overnight Express auto-
129 induction medium and BugBuster reagents were purchased from Merck KGaA
130 (Darmstadt, Germany). CDP from *Clostridium (Hungateiclostridium)*
131 *thermocellum* strain YM4 was initially provided by Prof. Momomitsu Kitaoka of
132 Niigata University, Japan. *E. coli* BL21 (DE3) competent cells were purchased
133 from Nippon Gene (Tokyo, Japan). C-Tube counter-diffusion (Otálora et al. 2009)
134 quartz capillaries were purchased from Confocal Sciences Inc. (Tokyo, Japan).

135 **Enzyme preparation.**

136 A gene coding Δ Cys-*Ct*CDP based on CDP from *C. thermocellum* strain YM4
137 (GenBank: AB061316.1) was designed, in which all 11 cysteine residues were
138 replaced with serine residues. None of the cysteine residues in *Ct*CDP are thought
139 to form disulfide bonds. This gene was codon-optimized for expression in *E. coli*
140 and synthesized by GenScript (NJ, US) with a 6x His tag at the C-terminal. It was
141 inserted into the pET-28b vector between the *Nco*I and *Xho*I sites with Ligation
142 High (Toyobo, Osaka, Japan). The vector was transformed into *E. coli* BL21
143 (DE3). Δ Cys-*Ct*CDP was expressed while transformed cells were cultivated in an
144 Erlenmeyer flask filled with 1 L of Overnight Express auto-induction medium at
145 30°C. After 18 hours of cultivation, the cells were collected by centrifugation, and
146 the crude enzyme was obtained after cell lysis with BugBuster reagents. The
147 crude enzyme was purified on a TALON his-tag cobalt affinity column (Clontech
148 Takara Bio USA, CA, US). The His-tagged target protein was eluted with a linear
149 gradient of 20 mM Tris-HCl buffer pH 7.5 containing 100 mM NaCl and 500 mM
150 imidazole. The His-tagged protein was then dialyzed against 20 mM Tris-HCl
151 buffer with an Amicon apparatus with 10,000 MWCO Biomax membrane filter
152 (Merck). Anion exchange chromatography with TOYOPEARL DEAE-650S
153 (Tosoh, Tokyo, Japan) was employed for further purification. Highly purified
154 Δ Cys-*Ct*CDP was eluted with a linear gradient of 20 mM Tris-HCl buffer pH 7.5
155 containing 250 mM NaCl and used for cellulose synthesis.

156 **Cellulose synthesis *in vitro*.**

157 0.10 $\mu\text{g/ml}$ $\Delta\text{Cys-CtCDP}$ and 10 mM cellobiose were introduced into a C-tube
158 counter-diffusion (Otálora et al. 2009) quartz capillary placed in 10 mM
159 cellobiose and 200 mM $\alpha\text{-G1P}$ solution three days before launch. The counter-
160 diffusion capillary consists of a 2 mm diameter quartz capillary and silicon tubing
161 containing agarose gel; this arrangement allows the outer solution to diffuse into
162 the capillary. Inside the counter-diffusion capillary, the initial $\alpha\text{-G1P}$
163 concentration was set to 0 mM and this gradually increased as $\alpha\text{-G1P}$ diffused
164 from the gel tube (Fig. 1A). The $\alpha\text{-G1P}$ concentration was controlled to minimize
165 the influence of gravity during cellulose synthesis before arrival at the ISS. The
166 Kirara service (JAMSS, Tokyo, Japan) was used to launch the experiment to the
167 ISS. The sample was kept in the microgravity environment of the ISS for one
168 month at 20 °C inside a thermostated box (Fig. 1B). The cellulose synthesized on
169 Earth was prepared similarly, except for the presence of gravity, as a control.

170 **WAXS measurements.**

171 WAXS measurements were done at the BL8S3 station of Aichi Synchrotron
172 Radiation Center (Aichi, Japan) with a 205.85 mm camera length. R-Axis IV++
173 (Rigaku, Tokyo, Japan) was used to record the diffraction, and radial integration
174 of diffraction intensity was performed with the program FIT2D (ESRF, Grenoble,
175 France). Sample capillaries were attached to the cell holder, and measurements
176 were conducted at the upper part (10 mm from the capillary top), middle part (14
177 mm from the capillary top), and bottom part (18 mm from the capillary top) of the
178 capillary (Fig. 1C).

179 Igor Pro (Wavemetrics, OR, US) was used to perform WAXS peak fit
180 analysis and to create graphics. FWHM (full width at half maximum) of peaks
181 assigned to the 020 plane of cellulose II and peak areas were determined,
182 assuming that scattering due to water was smooth and would not form any peak.

183 **SAXS measurements.**

184 SAXS experiment was conducted at the BL8S3 station of Aichi Synchrotron
185 Radiation Center under the following conditions: diffraction of 0.92 Å X-rays was
186 recorded on an R-Axis IV++ at a camera length of 3975.85 mm. Radial
187 integration of diffraction intensity was performed with the program FIT2D.

Sample capillaries were attached to the cell holder, and measurements were done at the upper part (10 mm from the capillary top), middle part (14 mm from the capillary top), and bottom part (18 mm from the capillary top) of each sample capillary (Fig. 1C).

SAXS data was processed with ATSAS (Manalastas-Cantos et al. 2021) and SasView (<http://www.sasview.org/>). The SAXS data were analyzed after subtracting the scattering curve of the negative control solution containing 0.10 µg/ml CrCDP and 10 mM cellobiose in a C-tube capillary. Earlier electron microscopy and atomic microscopy observations showed that complex structural features can co-exist in one reaction system (Hiraishi et al. 2009; Pylkkänen et al. 2020), and therefore we used a unified power law equation for fitting the data (Beaucage 1995; Tajima et al. 2019).

$$I(Q) = background + \sum_{i=1}^2 \left[G_i \cdot \exp\left(-\frac{Q^2 \cdot Rg_i^2}{3}\right) + B_i \cdot \exp\left(-\frac{Q^2 \cdot Rg_{i+1}^2}{3}\right) \cdot \left(\frac{1}{Q_i^*}\right)^{P_i} \right] \quad (1)$$

$$Q_i^* = Q \left[\operatorname{erf}\left(\frac{Q \cdot Rg_i}{\sqrt{6}}\right) \right]^{-3} \quad (2)$$

Q, I(Q), R_g, G, B in equations (1) and (2) are scattering vector, intensity, radius of gyration for a particular scattering body, Guinier function, and Porod-type function, respectively. The scattering vector was defined as $Q = 4\pi/\lambda \sin\theta$, where 2θ is the scattering angle, and λ is the wavelength.

Observation with scanning electron microscopy (SEM).

The solvent in capillaries containing synthesized cellulose was replaced gradually with tert-butyl alcohol and then the cellulose samples were freeze-dried in a lyophilizer (FDU-1200, Eyela, Tokyo, Japan) and collected by breaking the capillaries with a cutting stone (Hampton Research, CA, US). Samples were coated with Pt-Pd, and SEM images were captured with an FE-SEM S-4800 (Hitachi, Tokyo, Japan) at 1 kV.

217 **Results and discussion**

218 **Enzyme preparation**

219 *Ct*CDP was found to be unstable and lost its activity over several weeks. Since
220 synthesis of cellulose on the ISS was planned for one month, improving the
221 stability of *Ct*CDP was the first challenge for this study. Alexander *et al.*
222 suggested that the oxidation state of cysteine residues negatively affects the
223 *Ct*CDP activity, and therefore, we designed Δ Cys-*Ct*CDP in which all 11 cysteine
224 residues are replaced with serine residues (Fig. 2). This Δ Cys-*Ct*CDP did not lose
225 activity for at least two months. Characterization of the mutated *Ct*CDP will be
226 reported elsewhere.

227

228 **Optical observation of cellulose synthesized in counter-diffusion** 229 **capillaries**

230 In the *Ct*CDP reaction using the counter-diffusion reaction vessel, the reaction
231 proceeds as the donor substrate, α -G1P, is supplied from the gel tube by diffusion
232 (Fig. 1A). In the sample capillaries, there was an unreacted region, where no
233 product exists, on the opposite side from the gel tube. This result suggests that the
234 enzymatic reaction proceeded sequentially from the site of the gel tube, regardless
235 of whether the reaction takes place in space or on the ground (Fig 3A and B).
236 However, the appearance of the cellulose synthesized under the two conditions
237 differed significantly.

238 The cellulose synthesized on the ISS had an overall homogeneous gel-like
239 appearance, and no aggregates could be seen (Fig 3A). However, on the ground,
240 the formation of larger aggregates was observed, and they were more abundant
241 near the base of the gel tube, i.e., in the direction of gravity (Fig 3B). The density
242 of cellulose crystals is approximately 1.5 g/cm³, and under typical aqueous
243 reaction conditions, the synthesized cellulose particles be expected to settle under
244 gravity. This settling would not occur in the microgravity environment in space,
245 suggesting that cellulose synthesis under microgravity prevents the formation of
246 visible highly ordered structures and aggregates, affording more homogeneous
247 cellulose crystals. In addition, the highly ordered structure of cellulose

248 synthesized under microgravity was sufficiently strong to withstand its weight
249 because no aggregation was observed after the return to the Earth.

250

251 **WAXS measurements**

252 The cellulose synthesized in space appeared homogeneous and gel-like. On the
253 other hand, it is known that the crystalline form of cellulose is affected by drying
254 and other factors, so it was necessary to leave the cellulose in the reaction
255 capillary to perform X-ray diffraction measurements. To identify the allomorphs
256 of cellulose synthesized under microgravity and on the ground, WAXS diffraction
257 measurements were conducted. The WAXS diagram is shown in Fig. 4. The
258 scattering intensity increased monotonically in the range of $5 \text{ nm}^{-1} < Q < 16 \text{ nm}^{-1}$
259 due to the presence of an excess amount of water. All measurements showed
260 similar trends (Fig. 4). However, the scattering intensities of cellulose synthesized
261 in space were similar along the height direction of the capillary, in contrast to the
262 scattering intensities of cellulose synthesized on the ground, where the upper part
263 showed higher scattering intensity in all ranges ($5 \text{ nm}^{-1} < Q < 16 \text{ nm}^{-1}$). This
264 suggests that cellulose synthesized in space has a more uniform crystal size or
265 more uniform crystal orientation in the height direction of the capillary than
266 cellulose synthesized on the ground.

267 As shown in Fig. 4, three peaks were detectable in the range of $5 \text{ nm}^{-1} < Q <$
268 16 nm^{-1} . There were weak peaks in the WAXS diagram of cellulose synthesized in
269 space, whereas cellulose synthesized on the ground showed sharp peaks.
270 According to the formula $d = 2\pi/Q$, which describes the relationship between
271 scattering vector (Q) and real space (d), peaks of $Q = 8.69, 14.1$, and 15.6 nm^{-1}
272 correspond to real space $d = 7.23, 4.45$ and $4.03 \text{ \AA}$, respectively. A combination of
273 those d -values was matched with lattice spaces in the 110 , 110 , and 020 planes of
274 cellulose II, respectively (Kobayashi et al. 2011; French 2014). Therefore,
275 celluloses synthesized on the ground and under microgravity were both assigned
276 as crystalline cellulose II. Thus, gravity did not appear to influence the
277 polymorphic form of the product.

278 The areas and FWHMs of peaks attributed to the 020 plane in Fig. 4 were
279 determined and are summarized in Table 1. The 020 plane areas of ground-
280 synthesized cellulose II were larger than those of space-synthesized cellulose. The

281 average peak area of cellulose on the ground was twice as large as that of
282 cellulose synthesized in space, and the average FWHM was 10% smaller. Those
283 data suggest that cellulose synthesized in space has a smaller crystal size or
284 reduced degree of crystal orientation.

285

286 **SAXS measurements**

287 WAXS measurements confirmed that the cellulose synthesized in space was not
288 an amorphous gel, but consisted of particles of crystalline cellulose. Therefore,
289 SAXS measurements were carried out to obtain information on this particulate
290 cellulose.

291 Fig. 5 shows Log-Absolute SAXS plots and residual plots after subtraction
292 of scattering from the bottom part of each capillary. The residual plots indicate
293 that cellulose synthesized in space showed a more uniform density of particles
294 with various radii of gyration throughout the capillary than cellulose synthesized
295 on the ground (Fig. 5B and D). Specifically, there were more components in the
296 region of $Q < 1.0 \text{ nm}^{-1}$ in the upper and middle parts compared to the bottom part,
297 though there was no significant difference between the plots of the upper and
298 middle parts. Thus, the SAXS profile of cellulose synthesized on the ground
299 differed more depending on the position in the capillary, and the difference was
300 particularly pronounced in the region of $Q < 1.5 \text{ nm}^{-1}$, which means the particle
301 region with a radius of gyration R_g greater than 4.18 nm (Fig. 5C). As for
302 cellulose synthesized on the ground, scattering from the middle part of the
303 capillary was higher than scattering from other parts (Fig. 5C and D). Considering
304 that gravitational settling is the main cause of the variation in particle distribution
305 with capillary position, the observation of a higher density in the middle part of
306 the capillary seems strange. However, it might be explained by adsorption of
307 cellulose II lamellar crystals on the quartz glass during sedimentation and
308 aggregation. C7CDP-cellulose II lamellar crystals have a large hydrophilic area
309 with abundant hydroxyl groups on the surface (Hiraishi et al. 2009; Wada et al.
310 2021), and might therefore bind readily with SiO_2 at the surface of the capillary.

311 To further highlight the differences, a Kratky plot was performed (Fig. 6).
312 The scattering from the center of the ground-synthesized cellulose showed a clear
313 peak at $Q \approx 0.90 \text{ nm}^{-1}$, which is distinctly different from that of space-synthesized

cellulose. While the scattering intensity of cellulose synthesized on the ground varied with the height in the capillary, the scattering intensity of cellulose synthesized under microgravity was relatively homogeneous. These data qualitatively suggest that there was no significant difference in the number and volume of cellulose particle scatterers in the upper or bottom part of the capillary between the ground and space conditions. Nevertheless, there was a significant difference in the number and volume of scatterers in the middle part.

To quantitatively evaluate the size of the scatterers, we focused on the small-angle results in the SAXS measurements. We found that a unified power law equation (Beaucage 1995) gave a good fit, with sufficiently small values of χ^2/point for all parts of the capillaries (Fig. 7). Especially in the region of $0.07 \text{ nm}^{-1} < Q < 0.5 \text{ nm}^{-1}$, all SAXS scatterings were proportional to $Q^{-2.28} - Q^{-2.35}$, indicating that the particles have a thin plate shape, whether the cellulose is synthesized in space or on the ground (Kratky and Porod 1949; Pedersen 1997). This conclusion is consistent with previous studies showing that *CtCDP*-cellulose single crystals have a platelet shape (Hiraishi et al. 2009; Pylkkänen et al. 2020; Wada et al. 2021).

The small-angle region of the SAXS results did not show a good fit in Guinier plot analysis for all the samples. This suggests that all the samples obtained consist of a set of aggregates with multiple radii of gyration. Therefore, in this fitting analysis, we focused only on the radius of gyration R_{g2} , which corresponds to the peak at $Q \approx 0.9 \text{ nm}^{-1}$. Table 2 shows all the parameters of the fitting analysis; the average R_{g2} values for cellulose in space and on the ground were calculated to be $6.61 \pm 0.09 \text{ nm}$ and $4.57 \pm 0.84 \text{ nm}$, respectively. It has been shown that cellulose synthesized *in vitro* by *CtCDP* under batch conditions on the ground has a degree of polymerization of 9 and forms plate-like crystals with a thickness of about 5 nm (Hiraishi et al. 2009). This value is similar to the R_{g2} values of cellulose in space and on the ground. Thus, *CtCDP*-cellulose's crystalline lamellar structure existed in celluloses synthesized both in space and on the ground.

The parameter B_2 in Table 2 represents the number or density of particles having a radius of gyration R_{g2} . Cellulose synthesized in space had uniform B_2 values at all measured points (1.32, 1.34, and 1.27 for the capillary's upper, middle, and bottom parts, respectively). In contrast, cellulose on the ground had

different values (1.42, 4.18, and 1.94 for the upper, middle, and bottom parts of the capillary). This difference suggested that cellulose synthesized in space has a quantitatively more uniform density of particles with a radius of gyration R_{g2} in the height direction of capillary, as compared with cellulose on the ground.

Observation with SEM

Typical SEM images are shown in Fig. 8. Cellulose synthesized in space (Fig. 8A and B) had a finer network structure than cellulose synthesized on the ground (Fig. 8C and D). The network consisted of ribbon-like structures, which were estimated to be 100-200 nm wide in space-synthesized cellulose (Fig. 8B). In contrast, cellulose synthesized on the ground contained thicker aggregates with micrometer scale width (Fig. 8D), i.e., several times larger. The width of the thin ribbon-like structures synthesized in space was consistent with previous TEM and AFM observations of CDP-synthesized crystalline cellulose II (Hiraishi et al. 2009; Pylkkänen et al. 2020). This indicates that space-cellulose's ribbon-like structure was comprised of single to several cellulose crystals, while ribbons of cellulose synthesized on the ground contained more crystals. These results and the WAXS peak intensities suggest that the thick aggregated form might assemble through orientation or crystallization. These partial features of the network structure are consistent with the sparse (Fig. 8A and B) and dense (Fig. 8C and D) micrometer-scale appearance of cellulose network structure. The scale of these ribbon widths is similar to the scale of the wavelength of the visible light; therefore, these features would affect the optical appearance (Fig. 3) as well.

It is well known that cellulose II synthesized by *Ct*CDP self-assembles into a network structure (Pylkkänen et al. 2020). Such cellulose II synthesized by *Ct*CDP on the ground was observed as white precipitates or aggregates in the earlier studies, in contrast to the gel-like appearance of space-synthesized cellulose (Fig. 3A). In previous attempts to create gel-like products with supermolecular network architecture on the ground, researchers have added nanocrystals of polymers such as polyethylene glycol and cellulose I_{β} to the reaction mixture for cellulose synthesis by *Ct*CDP (Hata et al. 2017, 2018) to serve as scaffolds. We believe the present report is the first to describe the production of pure cellulose II crystalline gel.

381 Our observations indicate that once the cellulose II network structure is
382 formed in space, the supermolecular structure is stable after return to Earth. A
383 relatively light polymer (PMMA, 1.2 g/cm³) was reported to form a network
384 structure of crystalline polymer through viscoelastic phase separation on the
385 ground, and gravity appeared to have a negligible influence for at least 12 hours
386 (Tsurusawa et al. 2017). Thus, it is possible that the effect of gravity on cellulose
387 arises because of the high specific gravity of cellulose compared with water.
388 Therefore, the microgravity environment in space may be essential for the
389 production of cellulose II crystalline gel.

390
391

392 **Conclusion**

393 In the present study, we investigated the possibility that gravity influences the
394 crystallization and formation of highly ordered structure of cellulose II. We found
395 that cellulose synthesized in space did not form aggregates like those of cellulose
396 synthesized on the ground. WAXS demonstrated that similar nano-scale
397 crystalline cellulose II packing occurred on the ground and in space. However, the
398 SAXS experiment showed that cellulose particles in a capillary had higher
399 homogeneity when synthesized in space. SEM observation showed that space-
400 synthesized cellulose had a fine supramolecular network structure on the
401 micrometer scale, and this was strong enough to survive after return to Earth.
402 These findings suggest that gravity influences aggregate formation during self-
403 assembly to form the network. In this work, a bottom-up synthesis of pure
404 cellulose II crystal gel was achieved for the first time. The physical properties of
405 this newly created cellulose II crystalline gel remain to be investigated.

406

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549 **Fig. 1.** Experimental settings. (a) The counter-diffusion capillary. (b) Thermostated box containing
 550 a counter-diffusion capillary for cellulose synthesis under a microgravity environment
 551 (©NAS/ESA, Photo was from
 552 [https://www.esa.int/ESA_Multimedia/Images/2021/01/ICE_Cube_commercial_COVID-](https://www.esa.int/ESA_Multimedia/Images/2021/01/ICE_Cube_commercial_COVID-19_experiment)
 553 [19_experiment](https://www.esa.int/ESA_Multimedia/Images/2021/01/ICE_Cube_commercial_COVID-19_experiment)). (c) Schematic illustration of wide-angle and small-angle X-ray scattering
 554 experiment on the counter-diffusion capillary.
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556

557 **Fig. 2.** Locations of mutations on Δ Cys-*CtCDP* are highlighted in yellow in (a). Images of *CtCDP*
 558 tertiary structure are based on *CtCDP* monomeric structure (PDB ID: 5NZ7, chain A). The
 559 distribution of cysteine-to-serine substitutions in the *CtCDP* protein sequence (984 amino acids) is
 560 shown in (b). A graphic of the tertiary structure of *CtCDP* was created with PyMOL (Schrödinger,
 561 NY, US).

Fig. 3. Optical observation of capillaries containing cellulose synthesized in a microgravity environment (a) and on the ground (b). Cellulose synthesized in a microgravity environment showed no apparent aggregation, unlike cellulose synthesized on the ground.

567

568 **Fig. 4.** WAXS diagram of cellulose synthesized in a microgravity environment (a) and on the
 569 ground (b). Scattering from the upper, middle, and bottom parts are depicted by dashed, dotted,
 570 and solid lines, respectively. Cellulose synthesized in a microgravity environment had more
 571 uniform and weaker diffraction peaks of cellulose II than cellulose synthesized on the ground.
 572 Each arrow shows the location of a peak corresponding to a lattice space of cellulose II.

573

Fig. 5. Experimental SAXS curves for *CtCDP*-cellulose and scattering differences in the height direction of the capillaries. SAXS profiles of cellulose synthesized in space and on the ground are shown in (a) and (b), respectively. The insets show residual scattering after subtraction of the scattering from the bottom part of capillaries. Scattering from the upper, middle, and bottom parts are depicted by dashed, dotted, and solid lines, respectively.

581

582 **Fig. 6.** A Kratky plot demonstrating the difference in *Ct*CDP-cellulose particle distribution in the
 583 height direction of the capillaries. Kratky plot of cellulose synthesized on the ground (black lines)
 584 and in a microgravity environment (gray lines). Scattering from the upper, middle, and bottom
 585 parts is depicted by dashed, dotted, and solid lines, respectively.

586

587

588 **Fig. 7.** Experimental SAXS profile of *C7*CDP-cellulose and fitting analysis with equation (1).
 589 Scattering of cellulose synthesized in space from the upper part (a), middle part (b), and the
 590 bottom part (c) and scattering of cellulose on the ground from the upper part (d), middle part (e),
 591 and the bottom part (f) are depicted in log-log plots. Fitted curves are shown as solid lines, and
 592 measured values are shown as gray circles in each figure. All SAXS data in the region $0.07 \text{ nm}^{-1} <$
 593 $Q < 0.5 \text{ nm}^{-1}$ are proportional to approximately $Q^{-2.3}$, indicating that the cellulose particles have a
 594 platelet shape.

595

596

597 **Fig. 8.** Typical SEM images of cellulose synthesized in space and on the ground. Images of
 598 cellulose synthesized in space were captured at x500 (a) and x2000 (b) magnification. (c) and (d)
 599 show images of cellulose synthesized on the ground at x500 and x2000 magnification,
 600 respectively. Cellulose synthesized under a microgravity environment generated a network
 601 consisting of thinner ribbons, while cellulose synthesized on the ground had a network structure
 602 with matrix-like thick ribbons.

Table 1. Comparison of cellulose synthesized in space and on the ground in terms of peak areas and FWHMs derived from the 020 plane. Peak fit was performed against WAXS profiles of the upper part, middle part, and bottom part of each capillary. Peak areas derived from the 020 plane of cellulose synthesized in space were relatively small, and FWHMs were rather large compared to those of cellulose synthesized on the ground. The unit of area is arbitrary.

	cellulose synthesized in space			cellulose synthesized on the ground		
	upper part	middle part	bottom part	upper part	middle part	bottom part
Area	125±34	88.8±11.7	93.9±13.9	188±8	234±14	246±20
FWHM (°)	0.379±0.055	0.282±0.025	0.296±0.029	0.250±0.008	0.292±0.011	0.316±0.015
Average Area	103			222		
Average FWHM (°)	0.319			0.286		

Table 2. Parameters for fitting SAXS profiles to equation (1). Fitting analysis was performed for SAXS profiles of the upper part, middle part, and bottom part of each capillary.

	Cellulose synthesized in space			Cellulose synthesized on the ground		
	upper part	middle part	bottom part	upper part	middle part	bottom part
Chi ² /points	0.0849	0.0559	0.0525	0.0492	0.195	0.140
background	0.791±0.066	0.746±0.065	0.735±0.066	0.681±0.068	0.763±0.070	0.688±0.062
R _{g1} (nm)	34.4±0.7	35.5±0.7	34.4±0.8	33.5±0.5	32.7±0.2	32.4±0.2
P ₁	2.78±0.032	2.75±0.03	2.80±0.04	2.76±0.03	2.66±0.01	2.68±0.01
B ₁	0.619±0.051	0.67±0.05	0.526±0.056	0.721±0.058	1.23±0.02	0.943±0.021
G ₁	3360±125	3670±151	2980±134	3500±100	4700±53	3500±55
R _{g2} (nm)	6.48±0.41	6.56±0.41	6.78±0.45	6.22±0.44	3.50±0.00	4.00±0.00
P ₂	2.71±0.16	2.72±0.15	2.61±0.14	2.81±0.18	5.80±0.64	6.39±0.73
B ₂	1.32±0.10	1.34±0.10	1.27±0.10	1.42±0.10	4.18±0.32	1.94±0.09
G ₂	56.4±9.1	58.8±9.3	58.2±9.9	55.9±9.8	20.7±0.3	23.9±0.4