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Electronic Supplementary Information

Mesoscopic Heterogeneity in Nanocellulose-Containing Cell Storage Medium

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1. Materials.

A normal Roswell Park Memorial Institute (RPMI) 1640 medium purchased from Life Technologies Japan Ltd. was used as a basal medium. It contains phenolphthalein as a pH indicator. The specific resistance of purified water was greater than 18 MΩ·cm. For the particle tracking measurement, an aqueous dispersion of Fluoresbrite Yellow Green Microspheres, which were polystyrene (PS) particles containing a fluorescent dye, having a concentration of 2.5 wt% was purchased from Polysciences Inc. Diameters (*d*) of PS particles used were 1.1 ± 0.02, 3.1 ± 0.09, 5.8 ± 0.3, 10 ± 0.4, 19 ± 0.7 and 44 ± 0.9 μm (hereafter denoted as 1, 3, 6, 10, 20 and 45 μm, respectively). For the confocal laser scanning microscopic (CLSM) observation, Rhodamine 6G (R6G) purchased from Tokyo Chemical Industry Co., Ltd. was used as received. For the cell viability test, a LIVE/DEAD Viability/Cytotoxicity Kit, containing *O*,*O*'-diacetate tetrakis(acetoxymethyl) ester (calcein AM) and ethidium homodimer-1 (EthD-1), was purchased from Thermo Fisher Scientific Inc.

2. Preparation of NC-containing Medium.

An aqueous dispersion of NC with a concentration of 0.1 wt% was prepared from CEOLUS PH-101 (Asahi Kasei Co.), which was a crystalline cellulose powder, obtained by an aqueous counter collision (ACC) method.[S1] It was steam-sterilized using an autoclave LSX-300 (TOMY SEIKO Co., Ltd.) under a pressure of 0.2 MPa at 394 K for 15 min. Repeating the cycle of centrifugation and decantation, water in the NC dispersion was replaced by the RPMI 1640 medium. The resultant cell culture medium containing NC was sonicated by an ultrasonic bath, BRANSON 5510J MTH (Yamato Scientific Co., Ltd.) with a frequency of 40 kHz. After 1 hour, the NC medium was left undisturbed at room temperature for 72 hours (aging). The aging-induced change in the bulk viscosity was

monitored by an oscillation viscometer, Viscomate VM-10A (Sekonic Co.).

3. Particle Tracking Measurement.

The NC medium with and without sonication treatment was used. A portion of the PS particle dispersion was well-mixed into the NC medium. An aliquot of the dispersion was placed in a glass bottom dish (Matsunami Glass Ind., Ltd.) and it was sealed with a cover glass using vacuum grease. The sample was then left undisturbed for 30 min to equilibrate at room temperature prior to the observation. Our approach used for the particle tracking was based on an inverted microscope (ECLIPSE Ti, Nikon) with an NA 1.30 oil-immersion objective lens (Plan Fluor 100×, Nikon), as reported elsewhere.[S2,S3] For the measurement, a halogen lamp and a charge-coupled device (CCD) camera (DS-Qi1Mc, Nikon Instech Co., Ltd.) were used to illuminate the sample and acquire images of particles in the samples at a frame rate of 31 Hz, respectively. Each particle was monitored 10 times at 298 K to average the diffusion behavior.

4. Cell Suspension and Cell Viability Test.

Mouse fibroblast L929 (Cell Engineering Division, RIKEN BioResource Center) was used as a model cell. A 250 μL-cell suspension with a density of 2.0×10^5 cells·mL^{-1} was mixed with a 250 μL-NC medium with a concentration of 0.2 wt% in a conventional 24-well plate composed of tissue culture polystyrene (TCPS) (Corning Inc.). Cultures were maintained at 310 K (37 °C) in a humidified atmosphere containing 5% CO_2. After a given culturing time, the number and morphology of cells were evaluated using CLSM with a transmittance mode (C2+, Nikon Instech Co., Ltd.). A He-Ne laser with a wavelength of 405 nm was scanned on a plane in the sample and then the

transmitted light was detected through a 20x objective lens. Z-stack images with a size of 1 mm×1 mm were acquired from the liquid surface to the bottom of a medium with an interval of 10 μm between images. Such an image acquisition was made at five different locations in-plane in the same sample to take an average of them. To assess the cell viability, fluorescence images were acquired for cells stained with a LIVE/DEAD solution containing calcein-AM and EthD-1.[S4] A portion of a LIVE/DEAD solution containing calcein-AM and EthD-1 was added to the NC-containing medium obtained after cell culturing for 24 hours. The sample was left undisturbed at 310 K for 30 min in a humidified atmosphere containing 5% CO_2. The observations were performed using CLSM with GFP-B and TRITC filter blocks. The former block has a center wavelength/bandwidth (CWL/BW) = 470/40 nm for excitation and CWL/BW=535/50 nm for emission, while the latter has a CWL/BW = 540/25 nm for excitation and CWL/BW=605/55 nm/nm for emission.

5. Atomic Force Microscopic Measurement.

For Atomic Force Microscopic (AFM) observation, an aliquot of a diluted NC dispersion was gently placed on a freshly-cleaved mica substrate. The resultant substrate was dried at room temperature for 24 hours in a vacuum oven. AFM images were acquired by an E-sweep equipped with an SPI3800 controller (SII Nano Technology Inc.) using an intermittent contact mode at room temperature. A cantilever used was microfabricated from silicon. The spring constant and nominal tip radius were 14 N·m−1 and 10 nm, respectively. An Image J 1.48 software was used to analyze the morphology of NC. **Fig. S1** shows a representative AFM image for NC on the substrate. The average width and length averaged over 100 different NC were 21 ± 3 and 460 ± 200 nm, respectively. These were relatively unchanged after the sonication treatment.[S3]

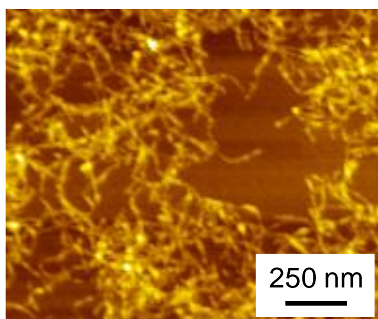

Fig. S1 AFM image for NC on a mica substrate.

6. Wide-angle X-ray Diffraction Measurement.

Wide-angle X-ray diffraction (WAXD) experiments were performed at the BL40B2 beamline of SPring-8 (Japan Synchrotron Radiation Research Institute). The wavelength of the incident X-rays and the sample-to-detector distance were 0.10 nm and 286 mm, respectively. The diffracted X-rays from a sample were recorded using a Rigaku R-AXIS IV+++ system (300 × 300 mm imaging plate). The NC dispersions with and without the sonication treatment were freeze-dried to obtain a NC powder. The NC powder in a Lindemann glass capillary was subjected to the WAXD measurement.

Fig. S2 shows one-dimensional diffraction profiles for NC powders obtained from (a) the original and (b) sonication-treated NC dispersions. Open symbols and solid lines denote experimental data and peak fitted curves based on a Gaussian function, respectively. For both samples, five distinct peaks were observed at a scattering vector (q) of 10.6, 11.8, 14.5, 16.0 and 24.2 nm^{-1}. These were assignable to the diffraction for ($1\bar{1}0$), (110), (102), (200) and (040) planes of cellulose I crystal, respectively.[S5,S6] A broad peak at q = 15.5 nm^{-1} was assignable to an amorphous halo.[S7] Apparent crystallinity of NC determined by the areal ratio of the crystalline peaks [S6,S7] was 65 %, regardless of the sonication. Thus, it is most likely that the crystallinity of NC remained unchanged even after the sonication treatment.

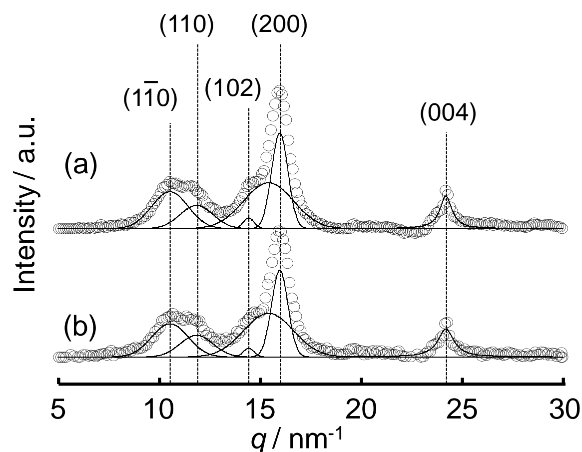

Fig. S2 One-dimensional WAXD profiles for the powders obtained from (a) the original and (b) sonication-treated NC dispersions.

7. Confocal Laser Scanning Microscopic Measurement.

The aggregation states of NC in the RPMI 1640 medium were examined by CLSM observation with R6G as a fluorescence probe. The adsorption of R6G on the NC surface gave a contrast in the CLSM image.[S8,S9] An NC medium containing R6G with a concentration of 10^{-5} M was prepared following a previously-reported procedure.[S3] CLSM images were obtained using a ZEN LSM700 microscope equipped with a semiconductor laser and an R6G filter block (Carl Zeiss Microscopy Co., Ltd.).

Fig. S3 shows the CLSM images for (a) the original NC medium and (b) one after sonication. For the original NC medium, denser and less-dense regions of NC were clearly observed, as evidenced by the presence of whiter and darker regions. The area of the denser regions apparently became larger after sonication. It should be noted that the density of the denser regions decreased after sonication due to a better dispersion state.

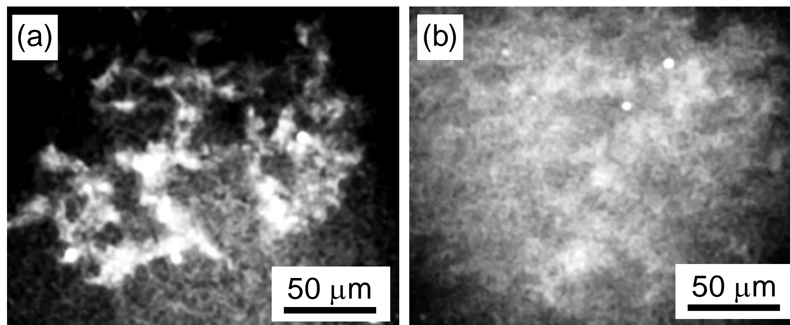

Fig. S3 CLSM images for (a) the original NC medium and (b) the medium after sonication treatment. The concentration of R6G, which was used as a fluoresence probe, was 10^{-5} M.

8. References.

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