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Production of Bacterial Cellulose with Well Oriented Fibril on PDMS Substrate

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Acetobacter xylinum, which is an aerobic bacterium, synthesizes bacterial cellulose (BC) with unoriented microfibril network in form of gel at the air and liquid interface (BC-air). In this work, we discovered that BC gel produced on an oxygen permeable substrate of polydimethylsiloxane (PDMS) shows strong birefringence with colorful images, indicating a liquid crystal-like structure. Furthermore, we found that uni-axially oriented BC gels can be obtained by culturing the BC on the PDMS with ridged morphology. The degree of orientation of BC gels, as revealed by the birefringence, increases with the decrease in the ridge size of the PDMS substrate. An optimum ridge size of 4.5 μm was observed where the BC gels show the highest birefringence (Δn), the highest fracture stress (σ), highest swelling degree (q), the lowest elastic modulus (E), and the thickest BC fibril. The optimum ridge size is related to the contour length of the bacteria cells. When the ridge size was smaller than 4.5 μm, the Δn steeply decreased to a value comparable to that on smooth flat PDMS mold surface. The fracture stress, σ of the uni-axially oriented BC gel under elongation was 4.6 MPa, which was 2.3 times higher than that of BC-air ($\sigma = 2$ MPa).

KEY WORDS: Gel / Bacterial Cellulose / Orientation / Polydimethylsiloxane / Mechanical Strength /

Cellulose is the most abundant biological macromolecule on the earth, which can be produced with several different pathways. The most popular way is isolation of cellulose from plants including separation process to remove lignin and hemicellulose.[1,2] Another way is the biosynthesis of cellulose by different types of microorganisms such as algae (Volania, Green, Red, and Brown), fungi (Saprolegnia, *Dictystelium discoideum*), or bacteria (Acetobacter, Achromobacter, Aerobacter, Agrobacterium, Pseudomonas, Rhizobium, Sarcina, Alcaligenes, Zoogloea).[3,4] But not all of these bacterial species are able to secrete the synthesized cellulose as fibrils extracellularly. Cellulose also can be produced by enzymatic *in vitro* synthesis starting from cellobiosyl fluoride[5,6] and chemosynthesis from glucose by ring-opening polymerization of benzylated and pivaloylated derivates.[7]

Bacteria such as *Acetobacter xylinum* (*A. xylinum*) which is aerobic bacterium under a static immersed cultivation conditions produce cellulose called bacterial cellulose (BC) with unoriented microfibril network in form of gel at the air and liquid interface (air/liquid) of the culture medium. This BC has high mechanical properties like tensile strength and modulus, high water holding capacity, high moldability, high crystallinity and also high biocompatibility.[8,9] Due to its unique properties, the BC has received much attention as a new functional material made from all natural ingredients in a wide range of medical, pharmaceutical, and prosthetic applications such as artificial skin[10,11] to heal wound,[12] membrane separation,[13] culture scaffolds for mammalian cells,[14] artificial blood vessel[15] and substrates for tissue engineering of cartilage.[16,17]

As high water holding capacity material, BC gel contains 90% or more of water. This water can be easily squeezed out from the BC gel by compressing with finger and the gel no more recoveries in the swelling property due to the hydrogen-bond formation between cellulose fibers. After removing of water by air drying, the BC gel will only rehydrate to the same low extent as that of plant cellulose after re-exposure to water, about 6%. However, after gently freeze-drying, it can absorb up to 70% of the original water content by re-swelling. In our previous paper, we reported on a BC-based hydrogel with a reversible swelling ability after repeated compression by incorporating natural polymer such as gelatin into BC to form a double network structure(BC-gelatin gel).[18] The fracture strength and the elastic modulus of the BC-gelatin gel under compressive stress were in the order of megapascals, which are almost equivalent to those of articular cartilage.[19]

Being concerned on its structure, production of the BC gel with well-oriented fibril arrangement could give benefit as substitute materials in medical and pharmaceutical applications. It is already well known that formation of highly oriented structure can improve the performance of materials. For example, oriented structure improves the stiffness and strength of crystalline polymers[20,21] compared with un-oriented materials. To date, limited attempts have been carried out to make an oriented BC fibrils during cultivation of bacterium *Acetobacter* by adding chemicals, such as lipid and polysaccharides,[22] or by using a surface with oriented polysaccharides chains.[23]

In the present study, we report a novel one-pot method to produce BC gel with highly oriented fibrils by cultivating BC

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on a substrate with ridge of various sizes. Taking advantage of its high oxygen permeability that is crucial for BC cultivation and its excellent moldability, polydimethylsiloxane (PDMS), a silicone based elastomer, is used as the substrate material. The BC gel was characterized by Scanning Electron Microscope (SEM) and Cross Polarizing Microscopy (CPM) and its mechanical properties were characterized by tensile strength measurement.

EXPERIMENTAL

Materials

Silpot 184 and silpot 184 catalyst used to form PDMS mold were purchased from Dow Corning Inc. Water proof abrasive paper (Sand paper) type DCC with varied grit size range from #240 to #2000 was purchased from Fuji Star, Sankyu Rikagaku Co., Ltd. Bacteria were used *Acetobacter xylinum*, American Type Culture Collection (ATCC) 53582. Culture medium was used based on Hestrin S and Schramm medium[24] (wt.-%); 5 g bacto peptone (difco bacto peptone, Becton Dickinson and Co, USA), 5 g yeast extracts (Difco Becton Dickinson and Co, Ltd), 2.7 g disodium hydrogen phosphate Na_2HPO_4 (Junsei Chemical Co. Ltd), 1.15 g citric acid (Wako Pure Chemical Ltd), and 20 g D (+) glucose (Wako Pure Chemical Industries, Ltd), and HCl. Aqueous solution of NaOH was used for purification.

Preparation of PDMS Mold

A ridged PDMS (*R*-PDMS) mold was prepared as follows: a polyethylene terephthalate(PET) plate, (10 cm × 10 cm), was rubbed manually in one direction by hand with sandpaper of various grit size (the bigger the grit code number, the smaller the grit size) gently for 10 times to produce a ridged PET template. After degassing, a mixing dope of Silpot 184 and its Silpot 184 catalyst (10:1 w/w) was placed onto the ridged PET template and covered with a smooth PET plate separated by a silicone spacer (1 mm in thickness) to form a reaction cell. Then the cell was heated at 60 °C for 4 h to form an R-PDMS mold of 1 mm in thickness. The ridge PET template can be reused many times to produce similar R-PDMS mold. The surface and the cross-sectional image of the R-PDMS mold were observed by using Scanning Electron Microscope (SEM). The average ridged size (R_a) of the R-PDMS mold was calculated from the ratio S/L, where S is the area between the roughness profile $r(x)$ of cross section image and its mean line and L is the evaluation length. As a control, smooth flat PDMS mold was prepared by the same procedure on PET template without rubbing process. Furthermore, PDMS mold with fine striped ridge was prepared on a micro-fabricated silicon wafer. The silicon wafer template was prepared as follows; a 40 mm × 40 mm of photo-mask with fine striped ridge (line and space size of 10 μm) was prepared by Emerson mask method. This mask was printed on oxide coated silicon wafer surface side. Unmask oxide coated silicon wafer surface was etched with HF buffer up to pure silicon surface. Then, by using 25% of tetramethyl ammonium hydroxide (TMAHi) the pure silicon surface was etched with different depth (2, 0.99, 0.53 μm).

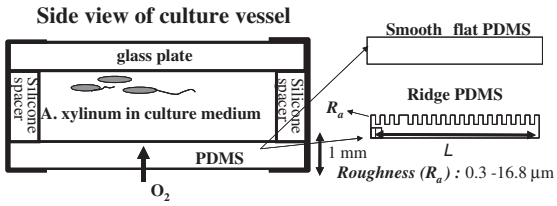

Figure 1. A schematic sealed culture vessel of BC produced on PDMS mold.

Finally, remaining SiO_2 was eliminated to obtain pure silicon wafer template.

Production of the BC on PDMS Mold

A sealed culture vessel was assembled by placing silicone spacers (1 mm in thickness) between the PDMS mold (thickness 1 mm) and a glass plate. After the culture vessel was tightly packed to avoid contamination, the *A. xylinum* was cultured in the vessel filled with Hestrin-Schramm medium at pH 6.0, and incubated at 28 °C in static condition for 7 d. The obtained BC gel about 6 cm × 6 cm × 1 mm in dimension was soaked into a large amount of distilled water for 1 d (the water was changed 3 times). After that, it was autoclaved at 121 °C for 20 min in aqueous NaOH 1% (%, w/v) to remove the alkali-soluble components, and then soaked into distilled water for a prolonged time to pH 7 and stored in distilled water at room temperature prior to use. A schematic sealed culture vessel is shown in Figure 1.

Measurement

The amount of water contained in the BC gel was characterized by the degree of swelling, q, which is defined as the weight ratio of as-prepared swollen BC gel to dry BC gel. Dry BC gel was obtained by drying the sample in vacuum until a constant weight. The degree of swelling, q represents the cellulose density in the BC gel.

Photo images were taken by using stereo microscope, Olympus SZX12. The BC gel was cut into 2 × 2 cm in size and directly place on the glass plate. Each side of BC gel was wiped with clean wipe-p (Asahi Kasei) prior to take photos.

Polarized light microscopy observation was performed using an Olympus BH-2. Samples of BC gel were placed on a precleaned micro slide glass plate (76 × 26 × (0.8–1.0) mm) (MATSUNAMI), and upper free surfaces were observed. The birefringence, $\Delta n = n_{parallel} - n_{perpendicular}$, of the BC gel was measured by a crossed polarizing microscope with Berek compensator.[25] The sample orientation direction was determined by turning the sample under the crossed polarizing microscope. Under these conditions, Δn was measured from retardation. The images were taken at 0, 45, 90, 135 and 180 degrees. Average Δn was determined by measuring Δn several times for each sample. During observation the sample are in wet conditions.

Morphology of the BC gel was studied by using SEM (HITACHI-S-2250N). Prior to observe with SEM, small pieces of BC gels were freeze dried (VirTis Advantage AD2. 0XL-

SC) for one day and mounted on SEM stub using double tip, and then sputter-coated with palladium (120 s) by ions sputtering (HITACHI-E 1010). The images was taken at 15 kV accelerate voltage with magnification 10 k.

The tensile strength tester (TENSILON, Orientec Co.) was used to measure the mechanical properties of BC gel. The wet samples of BC were cut into dumbbell shapes (dowel width, thickness and length of 2, 0.9, 12 mm, respectively) and stretched parallel to the orientation of the BC fibrils using a clamp attachment at a strain rate of 10% min^{-1} on the open air. The strain rate was referenced to the initial length of the specimens. Failure points of the tensile strength were determined from the peak of the stress-strain curve. The elastic modulus, E, was determined by the average slope over the strain ratio range of 0–10% from the stress-strain curve.

RESULTS AND DISCUSSION

PDMS is an oxygen permeable material that allows the aerobic bacterium of *A. xylinum* to proliferate and produce BC gel on its surface in a sealed culture vessel. The BC gel was grown 1 mm in thickness for 7 d in the culture vessel assembled with the PDMS mold and the glass plate. The BC gel formation was only observed from the PDMS mold surface and not from the glass surface, indicating that cells are localizing on the PDMS mold surface to obtain oxygen. It is reported that the BC production rate is proportional to the oxygen concentration in culture medium.[26] In this system, the production rate of the BC gel in the culture vessel (PDMS: 1 mm in thickness) was one-fifth of that under atmospheric condition, due to low oxygen concentration passed through the PDMS mold; the production rate of the BC gel increased with a decrease in the PDMS thickness (data not shown). The cellulose contents per unit volume in the BC produced at the PDMS/liquid interface was almost the same as that produced at the air/liquid interface (hereafter denotes as BC-air). Besides, semi-transparent nature of BC was also inherited in the BC produced on PDMS mold. However, the surface appearance of the BC on PDMS considerably differs from that of the BC-air.

The BC gel produced on both smooth flat PDMS mold (hereafter denotes as BC-PDMS) and on ridge PDMS mold (hereafter denotes as BC-R-PDMS) show a metallic glossy surface that comes from light interference (Figure 2a, column II and III). Especially, the BC-R-PDMS surface looks strongly metallic with straight lines along the ridged direction. This is in contrast to the un-glossy surface of the BC prepared between the liquid/air interfaces, BC-air (Figure 2a, column I).

Furthermore, only a weak birefringence is observed for BC-air (Figure 2b and 2c, column I), while the BC samples prepared on both PDMS mold show strong birefringence with colorful images when they are viewed under cross polarizing microscope (Figure 2b and 2c, column II and III). The birefringence of BC-PDMS is isotropic while the BC-R-PDMS is strongly anisotropic, exhibiting distinct dark and bright alternations during the sample rotation under cross polarizing microscope: a complete extinction is observed when the

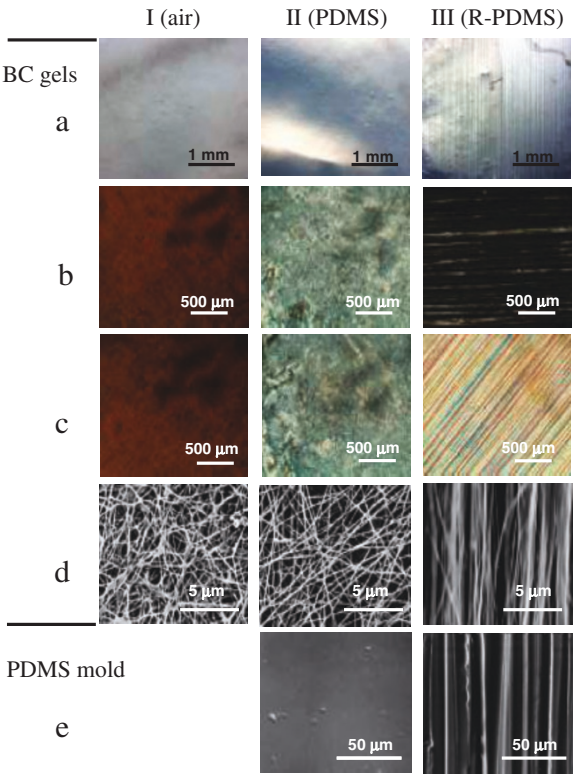

Figure 2. The bright field images (a), cross-polarizing microscope images at 0 degree (parallel to the substrate ridged direction) (b), and 45 degree (c), and SEM images (d) of BC gels produced at air/liquid (column I), PDMS/liquid (column II), and ridged PDMS/liquid (column III) interfaces. SEM images of PDMS and ridged PDMS mold are shown in (e) column II and III.

oriented direction is parallel or perpendicular to the polarizing direction, and the maximum brightness is observed when the oriented direction is 45 degree to the polarizing direction. It indicates a complete uni-axial orientation of the BC fibrils along the ridge of PDMS mold. This is reminiscent of the cellulose fibril orientation in plants. It is believed that polymerization, fibril formation, and orientation occur simultaneously in the plant cell and the plasma membrane inside the cell serves as a template for the synthesis and the orientation of cellulose fibrils.[27,28] However, it is rather surprising that the BC fibrils in the BC-PDMS show isotropic birefringence, indicating similarity to existence of randomly oriented in liquid crystal-like domains,[29] *i.e.* oriented BC fibrils lie in all possible directions.

The detailed observation by using SEM revealed width and orientation of the BC gel fibrils more clearly. Although it is difficult to estimate the exact size of each fibril accurately in limited view area after coating with palladium, we calculated an average width of these BC gel fibrils. The fibrils in the BC-PDMS have a wider and less ramified structure than that in the BC-air and partially aligned along a common axis (Figure 2d, column II and I). The average diameter of the BC-PDMS fibrils was 0.10 μm with a standard deviation of 0.04 μm, which was only slightly wider than that of fibrils in the BC-air (0.092 μm with a standard deviation of 0.03 μm). However, the average

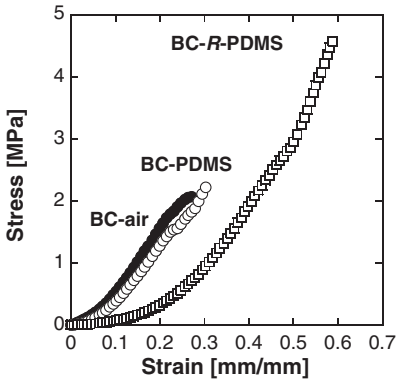

Figure 3. Tensile stress-strain curves of BC gels produced at air/liquid (●), PDMS/liquid (○), and ridged PDMS/liquid ($R_a = 4.5\,\mu m$) (□) interface.

width of fibrils on the BC-R-PDMS ($R_a = 4.5\,\mu m$) is 0.18 μm with a standard deviation of 0.03 μm, which is almost 2 times wider than that of fibrils in the BC-PDMS. These differences in the fibrils width may indicate the aggregation of the BC fibrils into wider parallel fibrils without forming a ramiform network on the PDMS surface. Further investigation is needed to clarify this assumption. In addition, it was reported that oxygen concentration does not affect the width of BC fibril but affects on its fibrils ramification.[26]

As mentioned above, the fibrils in the BC-PDMS are less ramified than that of BC-air and partially aligned along a common axis. Meanwhile, the fibrils in BC-R-PDMS are well aligned in uni-axial direction parallel to the ridge line direction of R-PDMS mold surface (Figure 2d, column III). The preferential orientation alignment of the BC fibrils may be similar to the formation of liquid crystal structure. Onsager predicted that a liquid crystalline phase appears in a solution of hard rod molecules above a threshold concentration, due to the parallel array of the rod molecules to decrease the excluded volume.[27] Localization of the aerobically growing bacterium of *A. xylinum*, which can be also regarded as a stiff rod [(0.6–0.8) μm × (1.0–4.0) μm], on the PDMS mold surface so as to obtain enough oxygen for BC production may help to exceed a critical concentration to form parallel array. Subsequently, the BC fibrils produced by well-oriented cell favors to form well-oriented alignment. Thus it was demonstrated that the solid/liquid interface plays an important role in the orientation of the BC fibrils.

The typical tensile stress-strain curves of the BC-R-PDMS (average roughness, $R_a = 4.5\,\mu m$), BC-PDMS, and BC-air, are shown in Figure 3. Stretching is applied in the direction parallel to the oriented BC fibrils for the BC-R-PDMS. One can see that the fracture stress of the BC-R-PDMS ($\sigma = 4.6$ MPa) increase 2.3 times compared with those of the BC-air ($\sigma = 2$ MPa) and BC-PDMS ($\sigma = 2.2$ MPa). However, a decreased elastic modulus of the BC-R-PDMS ($E = 0.84$ MPa) is simultaneously observed, which is about 3.8 times lower than that of BC-air ($E = 3.2$ MPa) and BC-PDMS ($E = 2.0$ MPa). When stretching is applied in the direction perpendicular to the

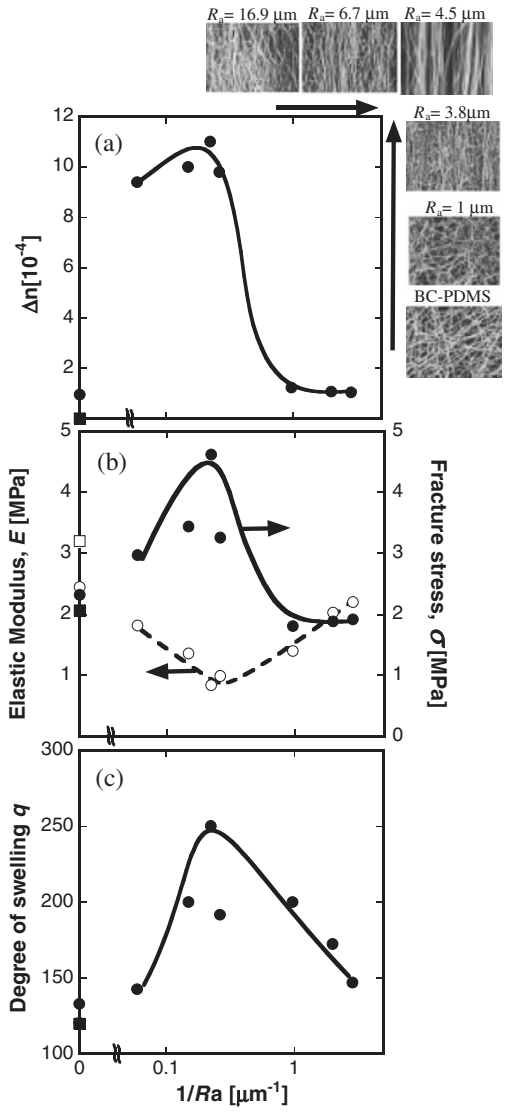

Figure 4. Dependence of birefringence Δn (a), elastic modulus E (●) and fracture stress σ (○) (b), and swelling degree q (c) of BC gels on PDMS substrate roughness $1/R_a$. The elongation was performed in the direction parallel to the oriented fibers. Data at $1/R_a = 0$ corresponds to the results obtained on flat PDMS surface. Results of BC gel produced at air/liquid interface are shown by (■, □) in the figures. The curve was shown to guide the eyes. The BC gel fibrils alignments are shown in the inserted SEM images in the Figure 4a. The arrows indicated increasing in the Δn.

oriented fibril for the BC-R-PDMS (data not shown), fracture stress and elastic modulus values are very much lower than that of in parallel stretching direction. This indicates that the BC-R-PDMS has anisotropy structure.

To study the effect of the ridge on the orientation and mechanical properties of the BC fibrils, we produced BC gels on the R-PDMS mold with various ridge sizes. The characteristic ridge size represented as an average roughness R_a (refer to the experimental section) ranging from 0.35 to 16.85 μm. The relationship between $1/R_a$ and the degree of orientation, Δn are shown in Figure 4a. The zero value of $1/R_a$ indicates the smooth flat PDMS mold surface. The Δn increases with the increase in $1/R_a$ when $1/R_a$ is lower than a critical value of

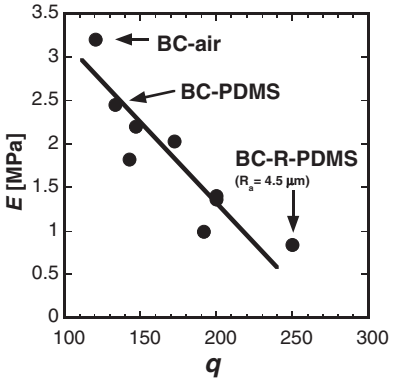

Figure 5. Effect of swelling degree, q on elastic modulus E of BC gel.

$0.22\,\mu m^{-1}$, corresponding to a critical roughness $R_a{}^c$ of 4.5 μm. When $1/R_a$ exceeds $1/R_a{}^c = 0.22\,\mu m^{-1}$, Δn steeply decreases to a value comparable to that on smooth flat PDMS mold surface. The critical $R_a{}^c$ of 4.5 μm approximately coincides with the length of *A. xylinum* (1.0–4.0 μm), indicating that for a rough surface with a ridge size smaller than the length of the cells, no ridge effect occurs and the orientation of the BC fibrils is similar to that on a smooth flat PDMS mold.

Dependence of fracture stress and elastic modulus (E) of BC-R-PDMS on PDMS mold roughness, $1/R_a$ is shown in Figure 4b. A drastic enhancement in the fracture stress of the BC gel is observed when $1/R_a$ is below $0.97\,\mu m^{-1}$ and the maximum fracture stress was given at $0.22\,\mu m^{-1}$ ($R_a = 4.5$ μm). Meanwhile, the elastic modulus (E) decreases gradually with increase in $1/R_a$ up to the critical value $1/R_a{}^c = 0.22$ μm^{-1} ($E = 0.84$ MPa), above which it increases and reaches 2.45 MPa, close to that of the BC-air. These profiles of the fracture stress and elastic modulus, E against $1/R_a$ well corresponds to the dependence of Δn on $1/R_a$, indicating that the mechanical strength of the BC gel is dominantly determined by the degree of orientation.

Furthermore, the water content revealed as the degree of swelling, q of BC-R-PDMS changes with $1/R_a$, and a maximum q was also observed at the critical roughness to give the highest Δn (Figure 4c). Since the mechanical property of hydrogel is commonly affected by the water contents, we also examined the q on the elastic modulus, E of those BC gels. As shown in Figure 5, the E decreases linearly with increase in the q. The E of the BC-R-PDMS is lower than that of BC-air and BC-PDMS. Lowering the elastic modulus of the BC-R-PDMS is mainly because of higher degree of swelling. The softening effect of high swelling exceeds the hardening effect of oriented fiber. Therefore, the decrease in E of BC gel is due to the increase in the water content of BC.

To investigate the real orientation effect on the fracture stress, we eliminate the effect of water content on the mechanical toughness of the BS gels by plotting a normalized fracture stress, $\sigma \times q$, against the normalized birefringence $\Delta n \times q$, (Figure 6a). One can see that at low $\Delta n \times q$, the $\sigma \times q$ increased slowly. However, at higher $\Delta n \times q$, the $\sigma \times q$ increased drastically. This is indicated that increasing in the fracture toughness was due to the enhancement in fibril orientation. In addition, a linear relationship between the normalized fracture stress and the width of the BC fibrils d is also observed as shown in Figure 6b. Thus, the increase in the fracture toughness is not only due to the enhancement in fibril orientation, but also due to an increase in the fibril width ness.

CONCLUSIONS

We succeeded in creating uni-axially oriented fibril of BC gels with improved mechanical toughness by culturing the cells on ridged PDMS surfaces. It was found that on a PDMS substrate with a ridge size about the contour length of the bacteria cells, the cells are able to produce BC gels with the highest birefringence (Δn), the highest fracture stress (σ), highest swelling degree (q), the lowest elastic modulus (E), and the thickest BC fibril.

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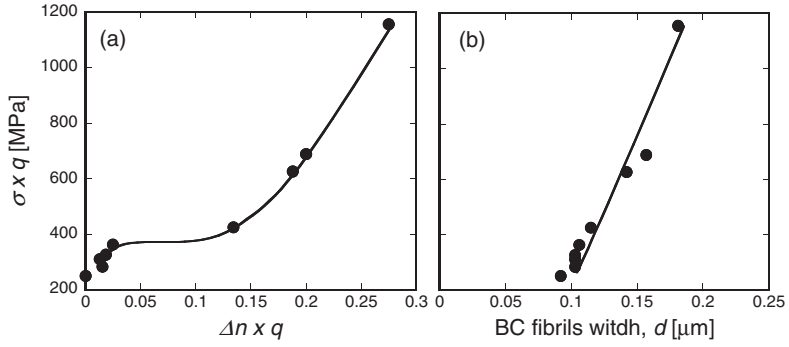

Figure 6. Dependence of $\sigma \times q$ on $\Delta n \times q$ (a), and BC fibril width, d (b).

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