



Title	A Facile Method to Fabricate Anisotropic Hydrogels with Perfectly Aligned Hierarchical Fibrous Structures
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A Facile Method to Fabricate Anisotropic Hydrogels with Perfectly Aligned Hierarchical Fibrous Structures --Manuscript Draft--

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Please submit a plain text version of your cover letter here. If you are submitting a revision of your manuscript, please do not overwrite your original cover letter. There is an opportunity for you to provide your responses to the reviewers later; please do not add them here.	August 29, 2017 Peter Gregory Editor-in-Chief Advanced Materials Dear Professor Gregory, I submit herewith a manuscript entitled 'A Facile Method to Fabricate Anisotropic Hydrogels with Perfectly Aligned Hierarchical Fibrous Structures', by Md. Tariful Islam Mredha, Takayuki Nonoyama, Tasuku Nakajima, Yun Zhou Guo, Takayuki Kurokawa and me for publication as a communication in the Advanced Materials. In this research work, we introduced a very facile novel method (drying a low density physical hydrogel in confined condition), by which we have been able to make perfectly aligned fibrous hydrogel with biomimetic hierarchical structures. By this method, we can induce sufficiently high internal force to align the polymer chains and simultaneously, can achieve fiber forming high concentration of polymers through drying process. We can control the bottom-up growth of polymers from the nano-to-submicron-to-micro scales in the macro-sized hydrogel. The method is useful for relatively rigid polymers such as alginate, cellulose etc. The mechanical and structural properties are comparable with natural ligaments. We can tune structure, alignment and toughness of the material within wide window by controlling its internal supramolecular interactions. The study would be a breakthrough in materials science for designing hydrogels with highly ordered hierarchical structures, which may find various bio-applications in future. This work is original. Any content of this manuscript in any form has not been published, not under consideration for publication elsewhere, and its submission for publication has been approved by all of the authors. May I request you to take necessary steps for publishing this article in Advanced Materials. I look forward to hearing from you with the hope of its rapid publication. Sincerely, Jian Ping Gong Distinguished Professor Laboratory of Soft & Wet Matter Faculty of Advanced Life Science, Hokkaido University & Director

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Abstract:	Anisotropic hydrogels with perfectly aligned hierarchical fibrous structures are fabricated by a simple method. Drying a physical hydrogel by confining the gel at its length direction can generate one-dimensional strong mechanical signal and tune the supramolecular interactions among the polymers to create hydrogels with highly aligned self-templated hierarchal structure similar to the collagen fibril structure in tendon/ligament. We can control the bottom-up growth of polymers from the nano-to-submicron-to-micro scales in the macro-sized hydrogel. The method is useful for relatively rigid polymers such as, alginate, cellulose etc. We can tune structure, alignment and toughness of the material within wide window by controlling its internal supramolecular interactions. The mechanical and structural properties are comparable with natural ligaments. The study would be a breakthrough in materials science for designing hydrogels with highly ordered hierarchical structures, which may find various bio-applications in future.

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A Facile Method to Fabricate Anisotropic Hydrogels with Perfectly Aligned Hierarchical Fibrous Structures

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Many methods have been developed to make polymer fibers in dry or hydrogel form including wet/melt spinning, electrospinning, and microfluidic techniques, where polymer solutions or melts are drawn as fibers with diameters on the order of micrometers to sub-micrometers.^[1-8] Constructing an anisotropic bulk hydrogel containing hierarchical fibrous architecture is still a challenge. We aim to develop a facile method to fabricate anisotropic hydrogels with hierarchical fibrous architectures. A material with such a structure is expected to exhibit mechanical properties similar to those of load bearing natural tissues such as ligaments and tendons. We have to learn from nature to achieve this. During tissue morphogenesis, biopolymers such as collagen are secreted from cells and assembled into fibrils, which can arrange laterally to form self-templated dense structures with hierarchy from the nanoscopic to the macroscopic length scales.^[9,10] It has been shown that both complex mechanical signals from the cell and dynamic supramolecular interactions among biopolymers play important roles in the creation of self-templated hierarchical

1 architectures.^[11,12] The rigid and rich interaction sites such as charges and H-bonds of
2 biopolymers are indispensable for structure formation. A rigid chain is more susceptible than
3 a flexible chain to orient in response to a mechanical signal.^[13] Lateral interactions between
4 polymer chains stabilizes the oriented structure. Although each physical interaction is very
5 weak, the supramolecular interaction through the summation of directionally controlled
6 hierarchical architecture could be very strong.^[12] Inspired by nature, we propose a facile
7 method by which we can induce spontaneous mechanical signals and tune supramolecular
8 interactions among polymers to create hydrogels with highly aligned self-templated hierarchal
9 structures similar to those of the collagen fibril structure in tendons and ligaments. This type
10 of structure, to the author's knowledge, is observed for the first time. This type of material
11 with hierarchal structure has high potential in biomedical applications such as artificial
12 tendons and ligaments.
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31 Our facile method includes drying a piece of diluted physical hydrogel consisting of semi-
32 rigid/rigid polymers in air at a confining geometry, as shown in **Figure 1(I)**. This *drying in*
33 *confined condition* method is abbreviated DCC. During drying, the gel shrinks due to the
34 evaporation of water (**Figure 1(II)**). As its two ends are fixed, the width and thickness of the
35 hydrogel will shrink, but not the length. Therefore, the gel experiences tensile stress in the
36 length direction during drying, causing orientation of the polymer chains along the length
37 direction. When dried, the polymer concentration increases and above a critical concentration
38 the polymers form nanofibrils through supramolecular interactions (**Figure 1(III)**). Further
39 drying induces aggregation of nanofibrils to form thicker fibers. The process is repeated to
40 form the hierarchical fibrous structure of the gel (**Figure 1(III)**). The re-swollen gel maintains
41 the same structure due to the formation of stable supramolecular interactions (**Figure 1(IV)**).
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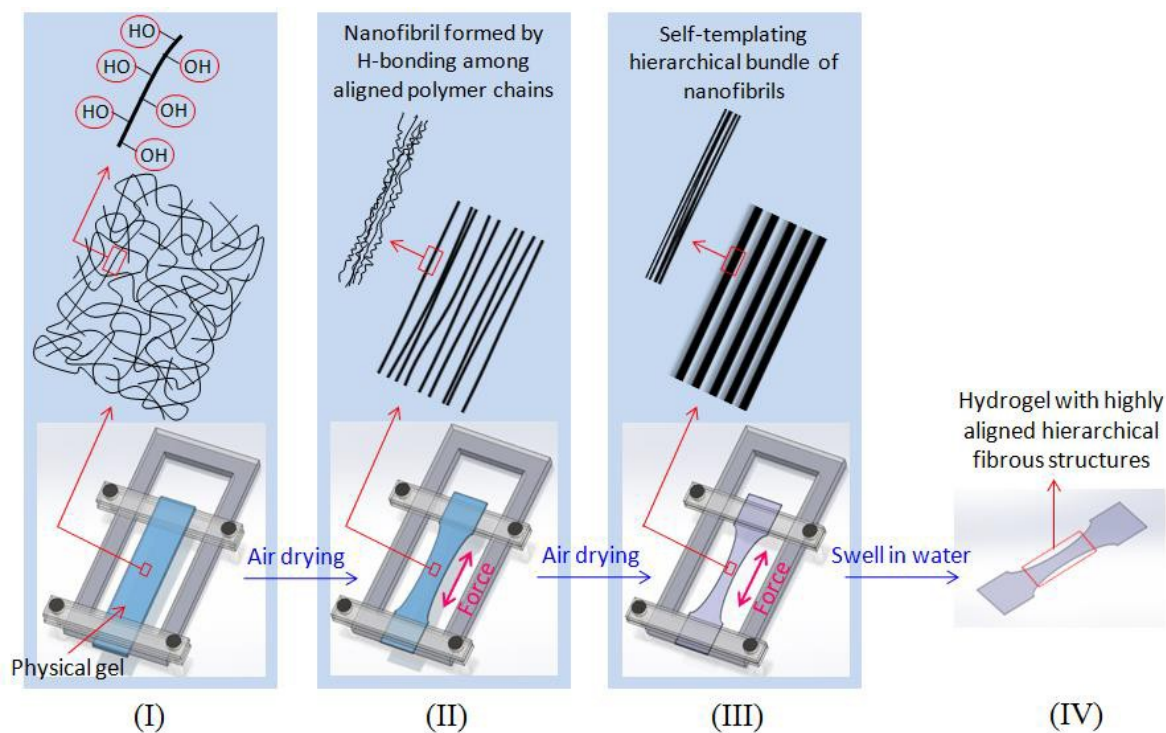


Figure 1. Design strategy. Schematic illustrations of DCC (drying in confined condition) method for creating perfectly aligned fibrous hydrogels with hierarchical superstructures. A rectangular piece of physical hydrogel (having fiber-forming H-bonding sites) is clamped to a sample holder at its two long ends (I). The gel is left to dry in air. Due to volume contraction during drying process, the gel's width and thickness shrink. As the shrinking in the length direction is restricted by the sample holder, the gel experiences tension along the length direction, which aligns the polymer chains in the length direction. When polymer density reaches the fiber forming concentration by drying, thin fibrils are formed along the tensile direction through H-bond formation (II). Further drying induces aggregation of nanofibrils to form thick fibers; this process is repeated to form the hierarchical fibrous structure of the gel (III). The re-swollen gel maintains the same structure due to the formation of stable H-bonds (IV).

Different rigid/semi-rigid linear polysaccharides such as alginate, cellulose, and chitin are critical to the morphogenesis and functionality of various hierarchically ordered natural materials.^[1,14,15] Alginate—a biodegradable negatively charged biopolymer originating from seaweed—bears structural similarity to glycosaminoglycan, one of the major components of the extracellular matrix.^[1] Alginate forms a physical hydrogel in the presence of divalent Ca^{2+} ions through ionic cross-linking.^[16] We chose Ca-alginate gel as one of the model physical gels to evaluate the potency of the DCC method. The ionic interactions can be sacrificed easily during the DCC process, which allows the polymers to align along the stress direction.

1 Being a polysaccharide, alginate has many hydrogen bonding sites, which could serve as the
2 driving force for fiber formation.
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7 To apply DCC method to an alginate system, Ca-alginate gel with diluted polymer
8 concentration was prepared by diffusion of 0.5 M Ca^{2+} into 4 wt% *aq.* Na-alginate solution
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10 **(Supporting Information, Figure S1)**. The gel shrunk slightly during gelation. The Ca-
11 alginate gel—1.5 mm thick and containing ~95 wt% water—was cut to rectangular shape of
12 size 10 mm × 50 mm, and its two long ends were clamped using a home-made holder, as
13 shown in **Figure 1(I)**. After clamping, the effective length (the part that was exposed to air) of
14 the sample was 30 mm. The gel was allowed to dry in air (temperature: 25 °C, humidity: 40–
15 60%). After complete air drying, the gel was re-swollen in water until it reached equilibrium.
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17 The final hydrogel prepared was termed DCC-alginate gel. The water contents of re-swollen
18 DCC-alginate gels were in the range 56–58 wt% (**Table 1**). This was very close to that of
19 natural ligaments (60–70 wt%).^[17] The air-dried alginate showed less rehydration ability than
20 the initial state, indicating the formation of stable hydrogen bonding among polymer chains
21 by drying. As shown by scanning electron microscopic (SEM) observations, though the
22 starting Ca-alginate gel had no oriented structure (**Figure S1**) a thick fibrous structure aligned
23 along axial direction was observed in the DCC-alginate gel (**Figure 2(a-i)**). Each microfibrer
24 was a bundle of several sub-micrometer-sized fibers (**Figure 2(a-ii, iii)**). The sub-micrometer-
25 sized fibers contained several nanometer-sized thin fibers (**Figure 2(a-iv, v)**). Thus, at least
26 three scales of hierarchical structure were identified. SEM image of the axial cross-section of
27 the DCC-alginate gel revealed a similar highly aligned hierarchical structure (**Figure 2(a-vi,**
28 **vii)**). We also prepared gels by applying 50% pre-stretching on the physical alginate gel while
29 performing DCC. This gel—termed 50%DCC-alginate gel—also showed three scales of
30 hierarchical structure (**Figure 2 (b-i, ii)**). The angle distribution of the microfibers of the
31 50%DCC-alginate gel was slightly sharper than that of the DCC-alginate gel (**Figure 2(c)**).
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1 The diameter distribution of the microfibers was narrower and the average diameter smaller
2 (**Figure 2(d)**). Birefringence of the 50%DCC-alginate gel—calculated from polarizing optical
3 microscopic (POM) observations—was higher than that of the DCC-alginate gel (**Figure S2**).
4 These results indicated that the microfibers were more aligned and smaller in size in the pre-
5 stretched gel. The results showed that we could tune the degree of alignment and
6
7 superstructure size by varying the degree of pre-stretching during DCC. We could not pre-
8 stretch more than 50% for alginate gel as the gel broke after some time during the DCC
9 process. Microfibers of both DCC-alginate and 50%DCC-alginate gels could be considered
10 *perfectly aligned* as the maximum deviation of fibers with respect to the axial direction was
11 only 2° (**Figure 2(c)**). To the best of the authors' knowledge, this was the first success in
12 obtaining alginate hydrogel with hierarchical fibrous structure and perfect alignment of fibers.
13 We investigated the structure of a gel prepared by normal drying (ND) (without confinement)
14 and re-swelling. The sample thus prepared was termed ND-alginate. The water content of
15 ND-alginate gel was similar to that of DCC-alginate gel (**Table 1**). No oriented
16 microstructure was observed in the ND-alginate gel (**Figure 2(e-i, ii)**).
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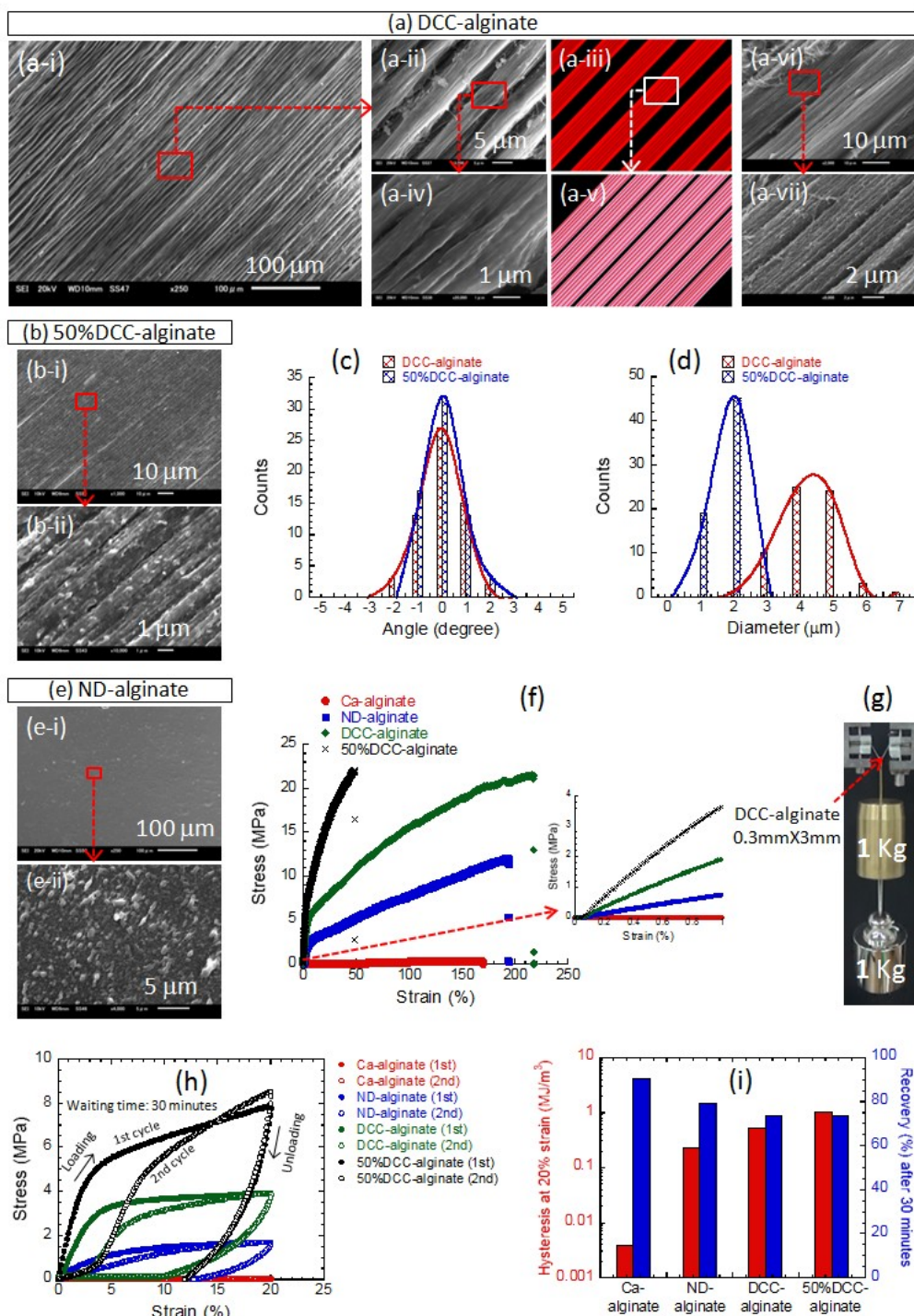


Figure 2. (a: i-vii) Structural analysis of DCC-alginate gel by SEM observation. Uniaxially aligned microfibers could be seen on the surface of DCC-alginate gel (a-i). Each microfiber was a bundle of several sub-micrometer-sized fibers (a-ii), as illustrated in (a-iii). Each of those sub-micrometer fibers was further composed of a nanofibrillar array (a-iv), which is illustrated in (a-v). SEM images of the axial cross-section of DCC-alginate gel indicated that fibrillar arrangements were retained in the bulk gel (a-vi, vii). (b-i, ii) SEM images of 50%DCC-alginate gel, where similar hierarchical structure was observed. (c, d) The distribution of microfiber angle with respect to the axial direction and the microfiber diameters of DCC-alginate and 50%DCC-alginate gels derived from (a-i) and (b-i), respectively, using image processing software. (e-i, ii) SEM images of ND-alginate gel, which

1 suggested that no fibrillar structure was formed. **(f)** Tensile stress-strain curves of alginate
2 hydrogels. Inset shows magnification of the low-strain region of the stress-strain curve. **(g)** An
3 image demonstrating the high load bearing capacity of the DCC-alginate hydrogel. **(h)** Cyclic
4 loading-unloading curves of alginate hydrogels. The waiting time between the first and the
5 second cycle was 30 min. **(i)** Hysteresis area and recovery—estimated from the ratio of the
6 second hysteresis area to the first hysteresis area. Tensile tests were performed at strain rate of
7 10 mm/min in air at 25 °C.
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10 The mechanical performances of gels prepared by the DCC method were better than those of
11 the ND-alginate and initial Ca-alginate gels (**Figure 2(f)**). **Figure 2(g)** shows that a tiny cord
12 of DCC-alginate gel (cross-section area: $\sim 0.3 \text{ mm} \times 3 \text{ mm}$) was able to bear a load of 2 kg
13 without breaking. Tensile experiments indicated that the Young's modulus (E), fracture stress
14 (σ_f), and fracture strain (ϵ_f) of the DCC-alginate gel increased significantly relative to the ND-
15 alginate gel (**Figure 2f**). The toughness of DCC-alginate gel—characterized by work of
16 extension (W)—increased significantly (**Table 1**). Modulus of the 50%DCC-alginate gel
17 increased further while the fracture stress maintained the similar value; fracture strain
18 decreased significantly relative to that of the DCC-alginate gel (**Figure 2f**). The aligned
19 superstructure in DCC-alginate gel led to increase in modulus and fracture stress due to the
20 formation of fiber structure. The increase in fracture strain of the DCC-alginate gel was
21 unique as the strength and extensibility usually change in opposite directions. Even though we
22 observed almost perfectly aligned microfibers, the nanofibrils were still not fully stretched.
23 When stretched, the nanofibrils might break bonding with other nanofibrils to show high
24 stretchability. Due to high degree of alignment in the 50%DCC-alginate gel, the nanofibril
25 could not stretch more, leading to fracture at low strain.
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52 Cyclic loading-unloading test was performed to find out if an energy dissipation mechanism
53 was involved in the deformation of alginate gels. Mechanical hysteresis was observed with all
54 the alginate hydrogels (**Figure 2(h, i)**), which indicated the presence of sacrificial bonds that
55 broke during deformation. At the same 20% strain, the hysteresis area increased with degree
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of fiber orientation. The DCC-alginate and 50%DCC-alginate showed hysteresis areas as high as 0.5 and 1 MJ/m³, respectively. After 30 min of waiting, about 75% of these hysteresis areas were recovered (**Figure 2(i)**). This indicated that the sacrificial bonds were almost reversible. Both ionic and hydrogen bonds serve as reversible sacrificial bonds to toughen the hydrogels.

Table 1. Mechanical properties and water contents of hydrogels prepared under different conditions. All the properties were measured after re-swelling in water. Data are presented as average value with standard deviation from three measurements. Data are compared with human knee ligaments.

Hydrogel name	Sample description	Young's modulus, E (MPa)	Fracture stress, σ_f (MPa)	Fracture strain, ϵ_f (%)	Work of extension, W (MJ/m ³)	Water (wt%)
Ca-alginate	Initial swollen sample	0.39 ± 0.05	0.32 ± 0.03	145.9 ± 23.3	0.24 ± 0.05	95
ND-alginate	Free dried	64.02 ± 14.35	10.26 ± 1.76	178.4 ± 14.7	11.78 ± 2.79	56
DCC-alginate	DCC without pre-stretching ^{#1}	203.3 ± 14.33	22.43 ± 0.93	201.4 ± 15.9	30.88 ± 1.97	57
50%DCC-alginate	DCC at 50% pre-stretching	367.35 ± 54.49	19.80 ± 2.24	51.1 ± 2.5	7.04 ± 0.45	56
p-Cellulose	Initial swollen sample	2.49 ± 0.21	1.58 ± 0.09	204.8 ± 11.0	1.48 ± 0.14	88
ND-cellulose	Free dried	19.20 ± 0.89	12.62 ± 0.40	407.7 ± 34.9	20.62 ± 2.18	59
DCC-cellulose	DCC without pre-stretching	59.0 ± 6.57	25.80 ± 1.33	267.3 ± 1.3	38.03 ± 2.83	58
50%DCC-cellulose	DCC at 50% pre-stretching	150.67 ± 11.79	39.33 ± 7.31	102.2 ± 9.0	32.04 ± 1.75	58
100%DCC-cellulose	DCC at 100% pre-stretching	342.44 ± 31.14	53.55 ± 0.53	34.7 ± 6.4	16.44 ± 0.99	58
Knee ligaments ^{#2}	-	65–447	13–46	11–44	-	60–70

^{#1}DCC: drying in confined condition.

^{#2}Human knee ligament data from medial collateral ligament (MCL), anterior cruciate ligament (ACL), and posterior cruciate ligament (PCL) are shown.^[17,18]

We further observed the progress of hierarchical fibrous structure formation of alginate gel *in situ* during DCC by POM observation (**Figure 3(a-c)**). Orientation strength (retardation value) increased linearly with drying time during the DCC process and reached equilibrium after a prolonged time of 300 min (**Figure 3(a, c)**). The air-dried gel showed slight re-swelling in water causing slight decrease in orientation strength, and then became stable (**Figure 3(b, c)**). These results indicated that the aligned microstructure of the gel was formed slowly as the DCC process progressed. The final structure remained stable upon re-swelling. The air-dried gel swelled in the width and thickness directions with dimension ratios of 1.32

and 1.26, respectively. The gel did not swell in the length dimension. The swelling only expanded the distance between fibers at a small scale without disturbing the orientation of fibers.

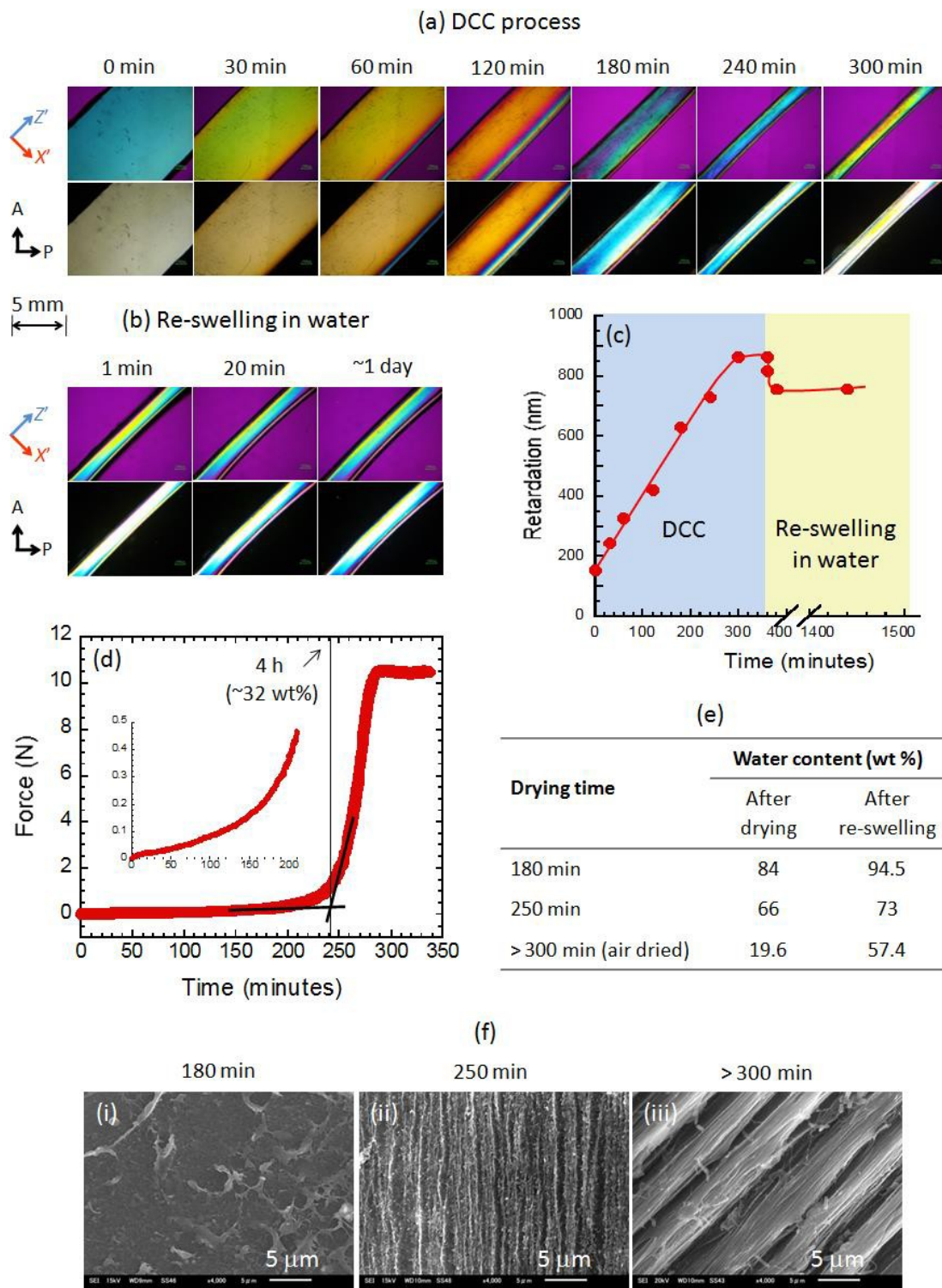


Figure 3. *In situ* observation of hierarchical fibrous structure formation of Ca-alginate hydrogel. (a) Polarizing optical microscopic (POM) images of Ca-alginate hydrogel during

1 DCC in the presence (upper row) and absence (lower row) of 530 nm tint plate. Sample at
2 initial state: water content = 95 wt%, thickness = 1.5 mm, width = 10 mm, length between
3 two confined ends = 30 mm. **(b)** POM images during reswelling of air-dried gel. All POM
4 images are shown at the same scale. A: analyzer, P: polarizer. X' and Z' : fast and slow axes of
5 the tint plate, respectively. **(c)** The variation in orientation degree (in terms of retardation
6 value) during DCC and reswelling. **(d)** Time profile of contractive force developed in the
7 alginate hydrogel along the axial direction during DCC. Inset shows magnification of the
8 initial part of the time profile. **(e)** Water content of the alginate hydrogel before and after re-
9 swelling with three different drying times (180 min, 250 min, and over 300 min) in the DCC
10 process. **(f)** Corresponding SEM images of these three samples after being re-swollen in water.
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14 To clarify the mechanism of oriented-structure formation, we measured the axial contraction
15 force of the gel generated during DCC process using a tensile tester. As shown in **Figure 3(d)**,
16 the contraction force increased gradually with time at the beginning of the drying process. It
17 increased abruptly at ~240 min when the polymer content increased to ~32 wt%. On further
18 drying, the contraction force saturated to a constant value of 10.55 N when the water content
19 of the gel decreased to an equilibrated value (19.6 wt%). From the dimensions of the dried
20 sample (size: 2.3 mm × 0.26 mm × 30 mm), a contraction stress of ~ 17.6 MPa was estimated.
21 We speculated that before the transition point (~240 min), interactions between polymer
22 chains were weak or negligible and the contraction force was related to the rubber elasticity of
23 the polymer chains. At the transition point (~240 min, where the contraction force abruptly
24 increased), polymers aggregated to form a stable hierarchical fibrous structure. To justify this
25 speculation, we prepared two DCC-alginate hydrogels by performing the DCC process for
26 180 min (before the transition point) and 250 min (after the transition point). The gels were
27 re-swelled in water and their structures observed by SEM. The gel prepared by the DCC
28 process for 180 min returned to its original shape and water content (**Figure 3(e)**) after re-
29 swelling and no oriented structure was identified (**Figure 3(f-i)**). The water content of DCC-
30 alginate gel prepared at 250 min did not change much after re-swelling (**Figure 3(e)**) and the
31 gel possessed aligned nanofibril structures (**Figure 3(f-ii)**). The re-swollen gel of the sample
32 with complete air-drying (time > 300 min) contained thick micrometer-sized fibers consisting
33 of nanofibrils (**Figure 3(f-iii)**). These results indicated that the abrupt transition in the force vs.
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1 time profile corresponded to the starting point for the formation of a hierarchical fibrous
2 structure.

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7 The above results showed that DCC drying produced different structures from normal
8 isotropic drying. In the DCC method, the gel was contracted along the width and thickness
9 directions. Contraction in length direction was prevented by clamping (**Table S1**). Due to
10 anisotropic contraction, the gel was effectively stretched along the length direction as drying
11 progressed, as shown by the large contraction force generated spontaneously in the axial
12 direction during DCC (**Figure 3(d)**). Due to this force, the polymer was progressively
13 oriented along the axis (**Figure 3(a, c)**). When the polymer density reached its fibril forming
14 concentration (~32 wt %, the inflection point of the force vs. time curve), the aligned polymer
15 chains became sufficiently close to each other to form stable nanofibrils. The nanofibrils
16 continued aggregating to form submicroscale fibers and the submicroscale fibers further
17 aggregated to form micrometer-scale fibers within the bulk hydrogel. The rigid nature of the
18 polysaccharide made the supramolecular interaction strong enough to prevent breaking upon
19 re-swelling.
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41 Stretching- and drying-induced orientations of polysaccharide hydrogel are not new
42 discoveries. Osorio-Madrado *et al.*^[19] described drying-induced alignment of cellulose
43 nanowhiskers in agarose hydrogels. Sehaqui *et al.*^[20] described the orientation of cellulose
44 nanofibrils caused by stretching and drying of its wet film. None of the methods produced
45 highly aligned hierarchical fibrous structures as in this work. A key difference between other
46 drying methods and our method was that, we applied DCC in a physical hydrogel with diluted
47 polymer concentration where the polymer chains were initially loosely cross-linked through
48 weak physical bonds. Upon drying via the DCC method, the relatively rigid polymer chains
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1 aligned easily along the tensile stress direction through the redistribution of physical
2 interactions to form an oriented superstructure.
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7 To verify the generality of the DCC method, we applied this method to another physical
8 hydrogel from cellulose. Unlike Ca-alginate gel, the physical cellulose (p-cellulose) gel was
9 solely based on H-bonding, which is a major supramolecular interaction found in biological
10 systems.^[12] In principle, H-bonding of a low density physical cellulose gel should be tunable
11 by the DCC method to create a superstructure similar to that of the alginate system. SEM
12 images indicated that ND-cellulose gel had no ordered microstructure (**Figure 4(a-i, ii)**). The
13 DCC-cellulose gel contained highly aligned microfibers consisting of multi-scale subunits of
14 submicro- and nanofibrils (**Figure 4(b-i, ii)**), similar to those of the DCC-alginate gel. The
15 results justified the applicability of the DCC method to different systems. Similar to the
16 alginate system, the diameter of the microfibers decreased with increasing pre-stretching
17 strain from 0% to 50% and 100% (**Figure 4(b-d)**). Nanofibrils in **Figure 4(d)** are much
18 extended than those in **Figure 4(b)**, indicating a higher degree of alignment in 100%DCC-
19 cellulose than that in DCC-cellulose. The mechanical properties of the gels were strongly
20 dependent on the degree of pre-stretching during DCC (**Figure 4(e)**). The strength (Young's
21 modulus and fracture stress) of the DCC-cellulose gels progressively increased with increase
22 in pre-stretching strain (**Table 1**). The extensibility (fracture strain) and toughness (work of
23 extension) progressively decreased with increase in pre-stretching (**Table 1**). Consistent with
24 the pre-stretching effect, the ND-cellulose that was dried in the free state without stretching
25 showed lower strength but higher extensibility than the DCC samples. The p-cellulose gel was
26 very soft in comparison to the samples that experienced drying treatment. Enhanced H-bond
27 formation led to the increase in the strength with the increase in pre-stretching, which was
28 similar to that observed in the alginate system. The decrease in the stretchability with the
29 increase in pre-stretching was attributed to the increased extension of the conformation of
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nanofibrils in the sample. The cellulose gels showed mechanical hysteresis and partial self-recovery, as shown in **Figure 4f**, indicating that the H-bonds served as reversible sacrificial bonds to dissipate energy in these gels. The increase in orientation led to increases in modulus and hysteresis area and decrease in recoverability (**Figure 4g**).

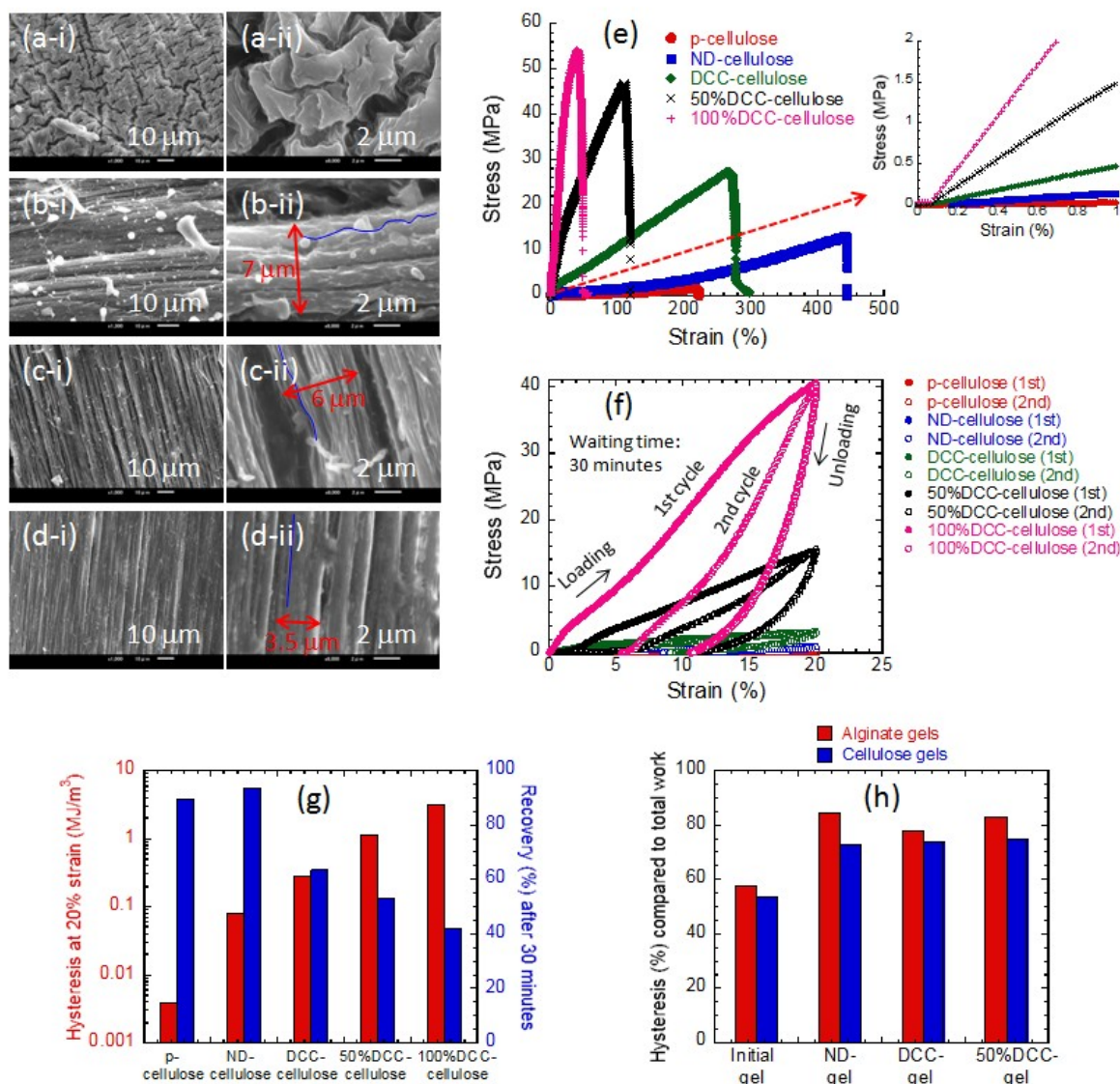


Figure 4. (a-d) SEM images of ND-cellulose gel (a-i, ii), DCC-cellulose gel (b-i, ii), 50%DCC-cellulose gel (c-i, ii), and 100%DCC-cellulose gel (d-i, ii). The size of microfibrils (measured by red arrow) decreased with increase in pre-stretching degree (b-ii, c-ii and d-ii). The blue lines in the images indicate the conformations of nanofibrils in the corresponding hydrogels. (e) Tensile stress-strain curves of different types of cellulose hydrogels. (f) Cyclic loading-unloading curves of cellulose hydrogels. The waiting time between the first and the second cycle was 30 min. (g) Hysteresis area and recovery—estimated from the ratio of the second hysteresis area to the first hysteresis area. Tensile tests were performed at strain rate

10 mm/min in air at 25 °C. **(h)** Comparison of the hysteresis area (%) of the first cycle (compared to the total work done by extension) for alginate and cellulose systems. The relative hysteresis area of cellulose gels was slightly lower than those of the corresponding alginate gels.

An interesting difference between cellulose and alginate systems was that fracture strain of the DCC-alginate gel increased relative to its non-oriented counterpart (ND-alginate) but pre-stretching led to less extensibility; while the fracture strain of the cellulose system decreased monotonously with stretching. This difference was attributed to the strength of the physical bonds. As revealed by studies on tough double network hydrogels^[21-22] and some other tough hydrogels,^[16,23-24] internal bond rupture during deformation caused an effect that delayed the fracture of materials by dissipating energy. The alginate system is based on both ionic and H-bonding. These bonds in alginate are less strong and can be ruptured by deformation, serving as reversible sacrificial bonds to toughen the hydrogel. Although the cellulose system was based only on H-bonding, the H-bonding in cellulose was very strong as seen in the poor solubility of cellulose fibers. As the degree of alignment increased, the H-bond of cellulose became too strong to rupture, leading to relative brittleness of the material. To confirm this argument, we compared the energy dissipation of the alginate system and the cellulose system by estimating the hysteresis area in relation to the total work for deformation. As shown in **Figure 4h**, the alginate gel system systematically showed larger ratio of hysteresis area to total work, indicating the presence of more sacrificial bonds in the alginate system. Maintaining a balance between structural and dynamic natures of sacrificial bonds was important to obtain both strength and toughness of the material. The mechanical properties of cellulose and alginate gels obtained by DCC were comparable with those of natural ligaments.^[18] This method offers an opportunity to tune the structural alignment and mechanical properties of a wide range of physical hydrogels.

1 We assumed that the rigidity of alginate and cellulose played important roles in sensing
2 mechanical signals effectively to form hierarchical superstructures. We applied the DCC
3 method to a physical polyvinyl alcohol (PVA) gel that had flexible polymer chains to justify
4 this assumption. PVA gel was solely based on H-bonding, as was the cellulose gel. The
5 persistence length of PVA was shorter than that of cellulose.^[25,26] As observed from SEM
6 images, no oriented structure was formed in 100%DCC-PVA gel (**Figure S3**). The result
7 indicated that the backbone rigidity of the polymer is an important criterion for the formation
8 of a hierarchical superstructure. This explained why hierarchical structures in nature mainly
9 consist of rigid biopolymers such as collagen and elastin.
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24 To conclude, drying a highly swollen physical hydrogel comprising rigid/semi-rigid polymer
25 in confined condition produced perfectly aligned fibrous hydrogel with biomimetic
26 hierarchical structure. During the confined drying process, sufficiently high tensile stress was
27 built to align the polymer chains and multi-scale fibrous structures (from nano- to submicro-
28 to microscale) were simultaneously formed in the bulk hydrogel via physical association. This
29 bottom-up growth of superstructures occurred only in relatively rigid polymers, but not soft
30 polymers. Interplay of strength and toughness of the material required proper bonding
31 strength between polymer chains to maintain a stable fibrous architecture and reversibly
32 dissipate energy by breaking and reforming bonds (sacrificial bonds). By controlling the
33 drying process with or without pre-stretching, oriented hierarchical structure and internal
34 supramolecular interactions could be tuned in a wide range, which influenced the strength and
35 toughness of the material. This study provides a general strategy for designing hydrogels with
36 highly ordered hierarchical structures, which may find bio-applications in future.
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58 **Experimental Section**

59 Experimental details are provided in the Supporting Information.
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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

Md.T.I.M. and J.P.G. conceived the idea, designed the study and wrote the paper. Md.T.I.M.

and Y.Z.G. performed the experiments. All the authors analyzed and discussed the results.

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References

- [1] N. Bhattarai, Z. Li, D. Edmondson, M. Zhang, *Adv. Mater.* **2006**, *18*, 1463–1467.
- [2] S. K. Chae, E. Kang, A. Khademhosseini, S. H. Lee, *Adv. Mater.* **2013**, *25*, 3071–3078.
- [3] E. Kang, Y. Y. Choi, S. K. Chae, J. H. Moon, J. Y. Chang, S. H. Lee, *Adv. Mater.* **2012**, *24*, 4271–4277.
- [4] Y. Cheng, F. Zheng, J. Lu, L. Shang, Z. Xie, Y. Zhao, Y. Chen, Z. Gu, *Adv. Mater.* **2014**, *26*, 5184–5190.
- [5] Q. Wu, N. Chen, L. Li, Q. Wang, *J. Appl. Polym. Sci.* **2012**, *124*, 421–428.
- [6] K. Yamaura, R. Kamakura, *J. Appl. Polym. Sci.* **2000**, *77*, 2872–2876.

- [7] J. S. Bach, F. Detrez, M. Cherkaoui, S. Cantournet, D. N. Ku, L. Corte, *J. Biomech.* **2013**, *46*, 1463-1470.
- [8] J. S. Bach, M. Cherkaoui, L. Corte, S. Cantournet, D. N. Ku, *J. Med. Dev.* **2012**, *6*, 045004:1-9.
- [9] Y. Wang, T. Azaïs, M. Robin, A. Vallée, C. Catania, P. Legriel, G. Pehau-Arnaudet, F. Babonneau, M. M. Giraud-Guille, N. Nassif, *Nat. Mater.* **2012**, *11*, 724-733.
- [10] E. G. Canty, K. E. Kadler, *J. Cell Sci.* **2005**, *118*, 1341-1353.
- [11] C. P. Heisenberg, Y. Bellaïche, *Cell* **2013**, *153*, 948-962.
- [12] M. J. Webber, E. A. Appel, E. W. Meijer, R. Langer, *Nat. Mater.* **2016**, *15*, 13-26.
- [13] R. Takahashi, Z. L. Wu, M. Arifuzzaman, T. Nonoyama, T. Nakajima, T. Kurokawa, J. P. Gong, *Nat. Commun.* **2014**, *5*, 4490.
- [14] A. C. Neville, *Biology of Fibrous Composites: Development Beyond the Cell Membrane* (Cambridge Univ. Press, 1993).
- [15] W. J. Chung, J. W. Oh, K. Kwak, B. Y. Lee, J. Meyer, E. Wang, A. Hexemer, S. W. Lee, *Nature* **2011**, *478*, 364-368.
- [16] J. Y. Sun, X. Zhao, W. R. K. Illeperuma, O. Chaudhuri, K. H. Oh, D. J. Mooney, J. J. Vlassak, Z. Suo, *Nature* **2012**, *489*, 133-136.
- [17] D. Amiel, E. Billings, Jr., W. H. Akenson, Ligament structure, chemistry, and physiology. In D. M. Daniel, W. H. Akeson, and J. J. O'Connor (editors), *Knee Ligaments: Structure, Function, Injury and Repair*, pages 77-91. Raven Press, New York, **1990**.
- [18] H. J. Jung, M. B. Fisher, S. L. Y. Woo, *Sports Med. Arthrosc. Rehabil. Ther. Technol.* **2009**, *1*, 1-17.
- [19] A. Osorio-Madrado, M. Eder, M. Rueggerberg, J. K. Pandey, M. J. Harrington, Y. Nishiyama, J. L. Putaux, C. Rochas, I. Burgert, *Biomacromolecules* **2012**, *13*, 850-856.

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- [20] H. Sehaqui, N. E. Mushi, S. Morimune, M. Salajkova, T. Nishino, L. A. Berglund, *ACS Appl. Mater. Interfaces* **2012**, *4*, 1043–1049.
- [21] J. P. Gong, Y. Katsuyama, T. Kurokawa, Y. Osada, *Adv. Mater.* **2003**, *15*, 1155-1158.
- [22] T. Nonoyama, S. Wada, R. Kiyama, N. Kitamura, Md. T. I. Mredha, X. Zhang, T. Kurokawa, T. Nakajima, Y. Takagi, K. Yasuda, J. P. Gong, *Adv. Mater.* **2016**, *28*, 6740-6745.
- [23] M. A. Haque, G. Kamita, T. Kurokawa, K. Tsujii, J. P. Gong, *Adv. Mater.* **2010**, *22*, 5110-5114.
- [24] T. L. Sun, T. Kurokawa, S. Kuroda, A. B. Ihsan, T. Akasaki, K. Sato, M. A. Haque, T. Nakajima, J. P. Gong, *Nat. Mater.* **2013**, *12*, 932-937.
- [25] L. M. J. Kroon-Batenburg, P. H. Kruiskamp, J. F. G. Vliegthart, J. Kroon, *J. Phys. Chem. B* **1997**, *101*, 8454-8459.
- [26] H. Li, W. Zhang, W. Xu, X. Zhang, *Macromolecules* **2000**, *33*, 465-469.

Table of content entry

Anisotropic hydrogels with perfectly aligned hierarchical fibrous structures were fabricated by a simple method. Drying a physical hydrogel by confining its length direction generated one-dimensional tensile force that controlled polymeric alignment and supramolecular interactions. We realized a tunable structure and mechanical properties in different types of rigid polymeric hydrogels and the properties were comparable with those of natural ligaments.

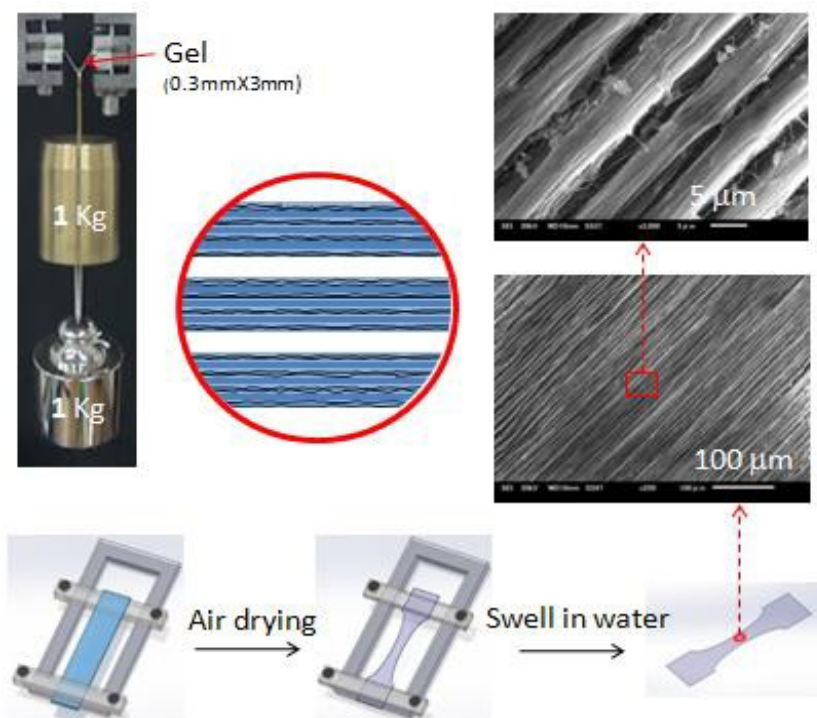
Keywords

Anisotropy, hierarchy, fiber, drying in confined condition, hydrogel

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Title

A Facile Method to Fabricate Anisotropic Hydrogels with Perfectly Aligned Hierarchical Fibrous Structures

ToC figure

Supporting Information

A Facile Method to Fabricate Anisotropic Hydrogels with Perfectly Aligned Hierarchical Fibrous Structures

Md. Tariful Islam Mredha¹, Yun Zhou Guo², Takayuki Nonoyama^{1,3}, Tasuku Nakajima^{1,3}, Takayuki Kurokawa^{1,3}, and Jian Ping Gong^{1,3*}

Materials and methods

Materials

Analytical grade Na-alginate (viscosity 80–120 cP, Wako Pure Chemical Industries Ltd., Japan), cellulose (Advantec filter paper, Toyo Roshi Kaisha Ltd. Japan), polyvinyl alcohol (PVA) (Nacalai Tesque Inc., Japan), calcium chloride (Wako Pure Chemical Industries Ltd., Japan), *N,N'*-dimethylacetamide (TCI, Japan), lithium chloride (TCI, Japan), acetone (Kanto Chemical Co. Inc., Japan), and ethanol (Nihon Hamby Ltd., Japan) were used as received without further purification. All aqueous solutions were prepared using ultrapure deionized water.

Preparation of alginate gels

Alginate solution was prepared by dissolving 4 wt% Na-alginate in water and stirring overnight. To prepare Ca-alginate gel, reaction cell was first prepared by sandwiching two glass plates (10 mm × 10 mm), separated using a 2 mm spacer. The upper end was kept open for solution insertion. Half of the reaction cell was filled with 4 wt% Na-alginate solution. 0.5 M CaCl₂ *aq.* solution was introduced from the upper empty part of reaction cell. Ca²⁺ ions diffused through the alginate solution. Ca-alginate gel started to form when alginate solution met Ca²⁺ ions. After complete gelation, Ca-alginate gel was taken out of the reaction cell and placed in 0.5 M *aq.* CaCl₂ solution for 1 d to complete the cross-linking process. Finally, the gel was thoroughly washed with water to remove non-cross-linked salt and polymer. The Ca-

alginate gel was used to prepare ND-alginate and DCC-alginate gels. ND-alginate gel was prepared by air drying and then re-swelling a piece of Ca-alginate gel. To apply DCC (drying in confined condition) on Ca-alginate gel, both ends of a sample of ~1.5 mm thickness and 10 mm width were clamped using the device shown in **Figure 1(I)**. The distance between the two clamps was 30 mm. The gel was left in the atmosphere (temperature: 25 °C, humidity: 40–60%) to dry. After complete air drying, the gel was taken out of the device, the clamped part cut from both ends, and the dried sample placed in water to re-swell. This re-swollen gel was denoted DCC-alginate gel. To prepare 50%DCC-alginate, the gel was initially stretched to 50% and maintained in the pre-stretched condition for air drying. After complete drying, the gel was re-swollen in water.

Preparation of cellulose gels

Physical cellulose gel (p-cellulose) was prepared from filter paper as per a previously reported^[1] procedure with slight modification. Cut filter paper sheets were sequentially washed with water and ethanol. The paper was activated by *N,N'*-dimethylacetamide (DMAc) for 12 h and then vacuum dried at 60 °C. To prepare cellulose solution, 0.75 g cellulose was dissolved in 100 g LiCl/DMAc (8 g : 92 g). Cellulose solution was cast into a glass mold of 3 mm thickness. The solution was left in air under ambient conditions (temperature 25 °C, humidity 40–60%). After 2 d, the solution became gel. The gel was first solvated in ethanol with two changes. The gel was equilibrated in water for a few days, changing the water several times to obtain p-cellulose gel (thickness ~1.5 mm). ND-cellulose gel was prepared by drying and re-swelling a piece of p-cellulose gel in air. DCC-cellulose, 50%DCC-cellulose, and 100%DCC-cellulose gels were prepared by applying DCC to p-cellulose gel (thickness: ~1.5 mm, width: 10 mm, length between two confined ends: 30 mm) with initial elongations of 0%, 50%, and 100%, respectively.

Preparation of PVA gels

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2 Physical PVA (p-PVA) hydrogel was prepared from PVA polymer according to a previously
3 described procedure.^[2] PVA ($M_w = 2000$ and saponification value $> 98\%$) solution (10 wt%)
4 was prepared in dimethyl sulfoxide/water (3:1, by weight) solvent by heating the solution at
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10 90 °C for 2 h. The solution was degassed in a vacuum oven and poured into a 2 mm thick
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12 glass mold. The glass mold was kept at low temperature (4 °C) for 12 h. Finally, the frozen
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14 hydrogel was placed in deionized water for 7 d with continuous exchange of water to yield the
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16 p-PVA gel. The p-PVA gel was dried in air, annealed at 135 °C for 4 h, and re-swelled in
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18 water to obtain ND-PVA gel. To prepare 100%DCC-PVA gel, a piece of p-PVA gel was
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21 dried in air under confined conditions (thickness: ~ 1.5 mm, width: 10 mm, length between
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23 two confined ends: 30 mm) with initial stretching of 100%. The dried gel was annealed at
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26 135 °C for 4 h and re-swelled in water.
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Structure

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33 The structures of the gels were characterized by polarizing optical microscopy (POM, Nikon,
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35 LV100POL) and scanning electron microscopy (SEM, JSM-6010LA, JEOL Ltd.). A color
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37 sensitized 530 nm tint plate was used for POM to distinguish polymer orientation. The
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39 orientation strengths of hydrogels were measured quantitatively from retardation values using
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41 a Berek compensator in POM. To prepare the sample for SEM observation, hydrated samples
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43 were freeze-dried using a freeze drying device (Advantage XL-70, VirTis freeze-dryer) and
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45 coated with gold using an ion-sputtering device (E-1010, Hitachi, Japan). The shapes and
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51 volumes of the samples were not changed after freeze drying.
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Water content

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58 The water contents of the gels were measured from the differences in weight of sample before
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61 and after freeze drying.
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Mechanical properties

Tensile tests of all the alginate and cellulose hydrogels were conducted in air at room temperature (25 °C) using a commercial mechanical tester (Autograph AG-X, Shimadzu Co., Japan). Rectangular gels ~10 mm in length were used. The tensile speed was 10 mm/min. Three samples were used for each tensile test. Cyclic loading-unloading test was carried out for alginate and cellulose gels up to 20% strain. The waiting time between cycles of each test was 30 min. The gel was coated with silicone oil to prevent air drying during the waiting period between cycles. Stress was defined as the force divided by the initial area of the sample cross-section. The work of extension was calculated from the area under the stress-strain curve.

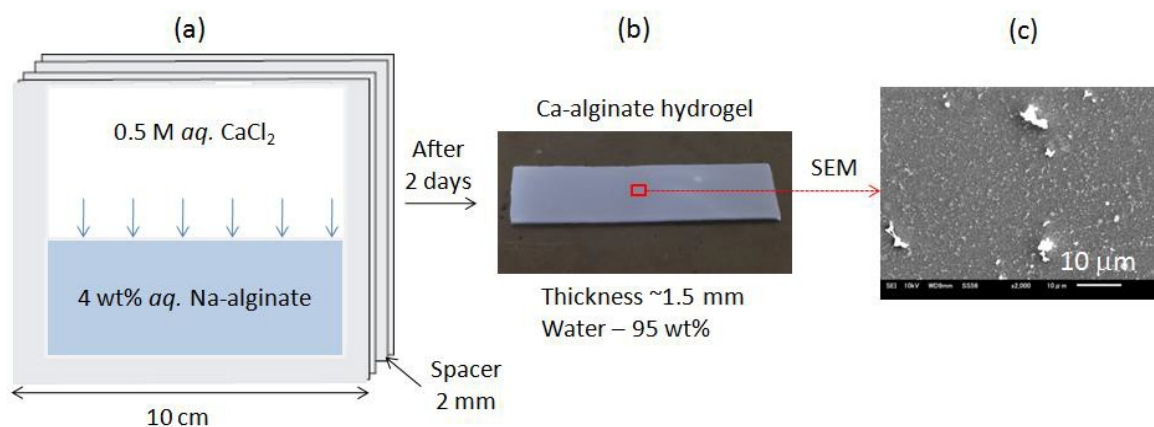


Figure S1. (a) Preparation of Ca-alginate hydrogel by diffusion of 0.5 M aq. Ca²⁺ ion through 4 wt% aq. Na-alginate solution. (b) Image of Ca-alginate hydrogel. (c) SEM image of Ca-alginate hydrogel.

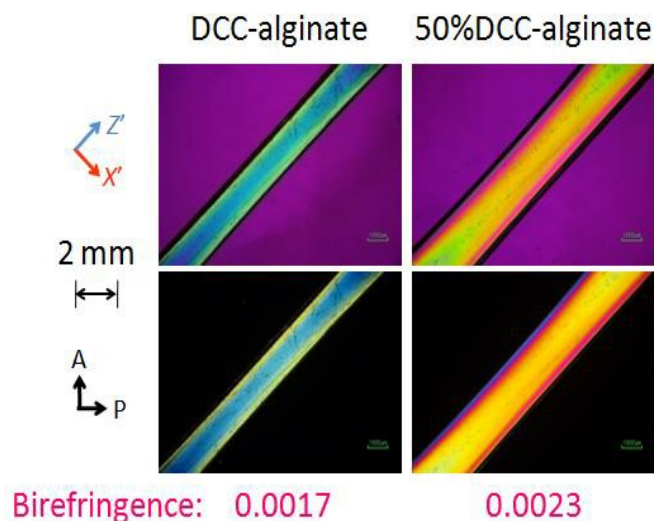


Figure S2. Polarizing optical microscopy (POM) images of DCC-alginate (1st column) and 50%DCC-alginate (2nd column) gels. Birefringence (3rd row)—calculated from the retardation value—indicates that polymers in 50%DCC-alginate gel were aligned to a greater degree than those in DCC-alginate gel. All POM images are shown at the same scale. A: analyzer, P: polarizer. X' and Z' : fast and slow axes of the tint plate, respectively.

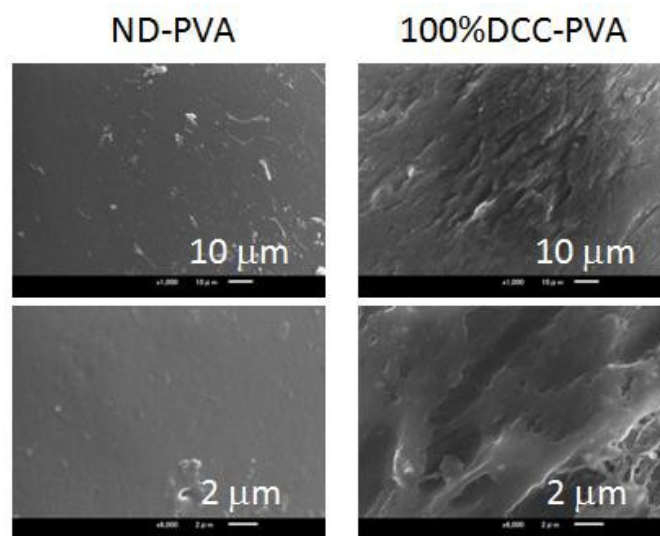


Figure S3. SEM images of ND-PVA gel and 100%DCC-PVA gel.

Table S1. Dimensional changes in length, width, and thickness directions of Ca-alginate gel (water content ~95 wt%) after DCC along the central axis.

Dimensions	Before drying	After drying	Contraction ratio in <i>DCC method</i> (ratio of final to initial dimension)
Length	30 mm	30 mm	1
Width	10 mm	2.3 mm	0.23
Thickness	1.5 mm	0.26 mm	0.17

References

- [1] W. Gindl, J. Keckes, *Polymer* **2005**, *46*, 10221.
- [2] S. H. Hyon, W. I. Cha, Y. Ikada, *Polym. Bull.* **1989**, *22*, 119.