Development of a Collagen Hydrogel with High Mechanical Strength by a Simple Orientation Method for Triple-helix

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Collagen hydrogels with oriented triple-helix were fabricated simply via the axial orientation of a dense collagen solution onto a glass plate and cross-linking. The orientation of the collagen triple-helix in the gels was clearly confirmed by X-ray diffraction measurements. The tensile strength of the oriented collagen gels in parallel was twofold higher than that of non-oriented gels as a control. Our collagen gels with oriented triple-helix can be useful as novel scaffolds in the tissue engineering field.

Collagen is the major fibrous protein responsible for the structural integrity of extracellular matrices (ECM) such as skin, bone, tendon, and other connective tissues. Its amino acid sequence is very characteristic: the presence of glycine as every third residue, and a high imino acid content, typically proline and hydroxyproline. The collagen molecule is known to have a rod-like triple-helical structure (diameter/length, 1.5 nm/300 nm) consisting of three polypeptide chains. Five triple-helical molecules further assemble to form a microfibril.[1] Furthermore, the micro-fibrils assemble to form collagen fibrils with 10–300 nm diameters.[2] In vivo, collagen fibrils organize into one direction to form collagen fibers of 0.5–3 μm in tendons or ligaments, and the orientation of these collagen molecules is known to be a significant factor in the mechanical strength of natural tissues such as bone, tendon, ligament, and the cornea. In other words, the molecular orientation in collagen matrices is important for strong mechanical properties. Accordingly, the construction of collagen hydrogels with oriented fibrils attracts much attention for tissue engineering. Recently, the preparation of collagen hydrogels with oriented fibrils using a magnetic field[3] or N_2 stream[4] was reported, but these methods required specific instruments or complicated manipulation. An effective and simple methodology to fabricate a collagen matrix with highly oriented collagen fibrils at the molecular level is strongly desired.

In this study, for the first time, we report a novel and simple method to prepare collagen gels with oriented triple-helix by the axial orientation of a dense collagen solution onto a glass plate and crosslinking processes. The mechanical strength of the collagen gel is significantly dependent on the orientation of triple-helix in the gel. Surprisingly, oriented gels in parallel show a twofold higher mechanical strength as compared to non-oriented gels as a control. This simple method to construct transparent collagen gels with oriented triple-helical molecules will have applications in the tissue engineering or biomedical fields.

Acid freeze-dried type I porcine atelocollagen powder containing 5% type III collagen was purchased from Nippon Meat Packers. Inc. A total of 168 mg of collagen was dissolved in 1 mL of 1 M acetate buffer at pH 3.6–4.0 (14 wt %) in a syringe for 24 h at 4 °C. Any air bubbles in the collagen solution were removed by centrifugation at 4 °C. The pH of the collagen solution was adjusted to 3.6–4.0 using 1.0 M NaOH under ice water using the syringe mixing system.[5] Two 180 μL aliquots of 5 wt % 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) solutions (EDC/NHS is 2:1) were added and mixed with the collagen solution. The final concentration of collagen was adjusted to 10 wt %, a high collagen concentration similar to native tissues. For the preparation of collagen gels with orientation of collagen solution (oriented gels), the reaction solution was poured onto a glass plate unidirectionally and subsequently covered with another glass plate for spreading the solution in the same direction (Figure 1). For the preparation of control gels without orientation of collagen solution (non-oriented gels), the reaction solution was poured onto a glass plate randomly. Silicone rubbers of 500 μm thickness were sandwiched between the glass plates to fix the thickness of the obtained gels. The glass plates containing the reaction solution were maintained for 24 h at 25 °C. The obtained gels were washed with phosphate buffered saline (PBS) at 4 °C for 24 h. Both hydrogels were punched out to obtain 8 mm diameter disks.

Optical photomicrographs and X-ray diffraction (XRD) patterns of the oriented and control collagen gels are shown in Figure 2. Both gels were extremely transparent. Interestingly, the shape of the oriented gel immediately changed to ellipsoid (major and minor lengths of 9 and 6 mm) after punching out using a round 8 mm diameter punch (Figure 2a), whereas the shape of the control gel did not change (Figure 2b). These shapes were maintained even after a half year of incubation in PBS at 4 °C. The typical shape change seemed to be due to the orientation of the collagen triple-helix in the gels. In order to clarify the orientation of triple-helix, the XRD patterns were recorded on an imaging plate (rapid-LS, Rigaku) at vertical direction on to the surface of the gels (Cu Kα radiation). The outermost diffraction (crossed position of blue lines) of both gels was estimated at 0.29 nm of the periodicity of the axial rise per residue. In contrast, the innermost strong diffraction (crossed position of red lines) was estimated at approximately 1.5 nm, the spacing of the intermolecular lateral packing of the collagen triple-helices (Figures 2c and 2d). In the case of Bovine skin treated with salt or alkaline solutions, a diffraction pattern was observed as a Debye–Scherrer ring.[6] In contrast, the XRD pattern of native tendon collagen shows a very narrow arc pattern.[7] Amazingly, the pattern of our oriented gel was similar to that of native tendon collagen compared with control gel which had less orientation of triple-helix. The results of the XRD patterns clearly suggested a highly ordered orientation of the collagen triple-helix in the

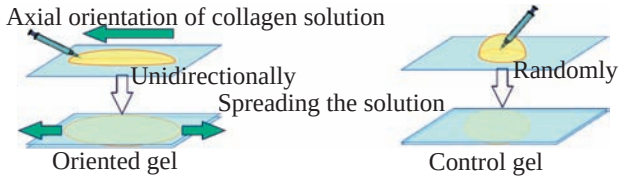

Figure 1. Schematic illustration of the fabrication process for oriented and control collagen gels.

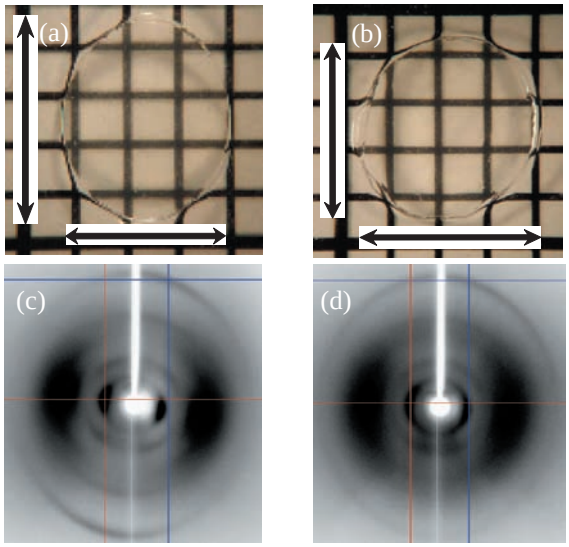

Figure 2. Optical photomicrographs and X-ray diffraction patterns of oriented (a and c) and control (b and d) collagen gels, respectively. Vertical and parallel scale bars are 9 and 6 mm (a) and 8 and 8 mm (b), respectively.

oriented gels as compared to the control gels. The direction of the collagen triple-helix in the gels was the same as the direction of orientation of the collagen solution spread onto a glass plate. We speculated that the axial orientation process of the reaction solution of high concentration (10 wt %) on the glass plate is important which caused this orientation of the collagen triple-helix because the other processes were exactly the same as the preparation of the control gel.

We evaluated the mechanical strength of both collagen gels because it is well known that the mechanical properties of a collagen matrix are strongly influenced by the orientation of the collagen triple-helix. The tensile strength of the oriented and control collagen gels was measured using a Shimazu EZ-test. Both types of collagen gels of 100-μm thickness were prepared, and the specimens (width/length/thickness, 10 mm/30 mm/100 μm) were used for the measurement of tensile strength. The oriented gels were pulled in the parallel and vertical directions with the direction of the collagen triple-helix. Figure 3 shows the tensile strength and breaking elongation of each sample and the typical stress/strain curves of each sample shown in Supporting Information.[8] No statistically significant differences were observed between the oriented gel pulled in vertical direction and the control gel in the tensile strength and breaking elongation. However, the tensile strength of the parallel direction was approximately twofold higher than that of the control gel, although the elongation properties of the oriented gel in parallel slightly decreased. To the best of our knowledge, it is the first report of controlling

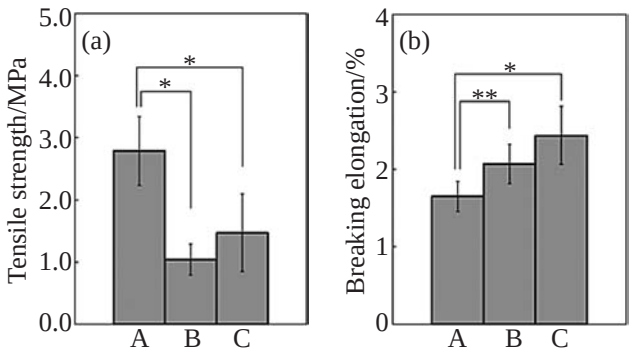

Figure 3. Tensile strength (a) and breaking elongation (b) of the oriented and control gels. The oriented gels were pulled in parallel (A) or vertically (B) with the direction of the collagen triple-helix. C is control gel. These are average values at more than three times. Statistically significant difference using two-sample *t* test ($^{*}P < 0.01$, $^{**}P < 0.05$).

the orientation of collagen triple-helix via a simple method and the resulting significant difference in mechanical strength due to the orientation of the collagen triple-helix.

In summary, collagen hydrogels with oriented triple-helix were successfully prepared by the axial orientation of a dense collagen solution onto a glass plate and crosslinking. The oriented gels underwent an interesting shape change due to the orientation of the collagen triple-helix. Furthermore, the mechanical properties of the oriented gels depended strongly on the orientation of triple-helix, and the tensile strength was twofold higher than that of the control gel. Transparent collagen hydrogels with oriented triple-helix will be useful as novel collagen materials for various tissue engineering fields such as bone, tendon, ligament, and cornea.

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8 Supporting Information is also available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

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