Mechanically Drawn Hydrogels Uniaxially Orient Hydroxyapatite Crystals and Cell Extension

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Freeze−thawed poly(vinyl alcohol) hydrogels were structurally oriented by uniaxial mechanical drawing up to 1−5 times their original length, and the orientation was fixed by successive chemical cross-linking using glutaraldehyde (20 mol %). Hydroxyapatite (HAp) crystals were successfully mineralized onto the oriented hydrogels by repeated alternate immersion into the solutions of phosphate ion and calcium ion. X-ray diffraction images confirmed that the *c*-axis of the HAp crystal cell was oriented perpendicularly to the surface of the hydrogel. In addition, polarized infrared spectroscopy confirmed the orientation of the phosphate ion. We investigated the effects of these structurally oriented HAp−PVA hydrogel hybrids on the anisotropy of cell extension. Scanning electron microscopy confirmed that mouse fibroblasts (L929) extended onto the oriented HAp−hydrogel hybrid along the drawing direction. These results may lead to the development of a tissue engineering scaffold for yielding oriented bony tissues.

Introduction

Hydroxyapatite ($Ca_5(PO_4)_3OH$, HAp) is an important bioceramic found in bone and tooth in living vertebrates and has excellent hard-tissue compatibility.[1,2] We recently developed a new method for the mineralization of HAp onto substrates such as poly(vinyl alcohol) (PVA), coating poly(ethylene) films,[3] and PVA hydrogels.[4−6] The PVA gel is immersed into an aqueous solution containing calcium ions to capture a certain amount of calcium ions, presumably through the chelation of the hydroxyls even after being washed with pure water. When the gel was successively immersed into an aqueous solution containing phosphate ions, the calcium ions bound with the phosphate ions to mineralize HAp on/in the gel. This process was repeated and formed HAp approximately 100 times faster than the conventional biomimetic process.[7] The amount of mineralized HAp increased with an increase in the number of treatment cycles. Thus, bone-like HAp−hydrogel hybrids have been developed facilely by the method of alternative immersion. The HAp−chitosan gel hybrids prepared by alternate immersion showed good biocompatibility with mouse fibroblast cells (L929 cells).[8] We have also reported that the HAp−agarose gel hybrids showed excellent hemostatic capabilities, using extraction bony defects in monkeys, and excellent effects on bone reconstruction in the mandible of adult monkeys.[9] These reports support the potential applications of these HAp−hydrogel hybrids to medical devices such as tissue reconstitution matrixes.

Most of the bony tissues in the vertebrates have a morphology uniaxially oriented along their longitudinal direction, giving anisotropy to their mechanical properties, which is crucial for supporting the living body.[10] This morphological characteristic of bone inspired us to develop the uniaxially oriented HAp−hydrogel composites with anisotropic biological properties. To our knowledge, cell extension directed by the controlled molecular orientation of the hydrogel substrate has never been studied. In this study, we attempted to mineralize HAp onto a PVA hydrogel oriented by mechanical drawing and found that the *c*-axis of the HAp crystal was oriented orthogonally to the drawing direction and that most of the longitudinal direction of the extended L929 cells was directed along the drawing direction.

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Experimental Section

Materials. PVA with a molecular weight of 88 000 (TCI) was used as received. The aqueous solution (25 wt %) of glutaraldehyde (GTA, WAKO Pure Chemicals Co. Ltd.) was used as the cross-linking agent and was used as received. Calcium chloride ($CaCl_2$, WAKO) and sodium hydrogen phosphate (Na_2HPO_4, WAKO) were used as received.

Hydrogel Preparation. The mechanically drawn PVA hydrogels were prepared as follows. An aqueous solution of PVA (10 wt %) was frozen at −20 °C, followed by thawing at 4 °C. The solution became more viscous than the untreated sample. When the freeze−thaw procedure was repeated 10 times, the solution transformed into a white elastic gel with macroscopically smooth surfaces and became very tough like silicon rubber. The PVA gel was cut into a rectangular sample (10 mm × 10 mm × 1 mm) that was drawn up to 1−5 times as long as its original length without breaking, immersed in an aqueous solution of the chemical cross-linking agent GTA (0.6−2.4 M) for 3 days, and then successively immersed in an acidic solution (HCl, pH = 2) for 1 day under the drawing conditions.

Alternate Immersion Treatment. The drawn hydrogels were punched into disks of 10 mm diameter. The disks were alternately immersed into aqueous solutions of $CaCl_2$ (0.2 M Ca^{2+}) and Na_2HPO_4 (0.12 M PO_4^{3-}) and were rinsed with pure water after each immersion. The details of this method have been described in previous reports.[4−6]

Cell Cultivation. The cell adhesion test of the drawn hydrogels immersed alternately in Ca^{2+} and PO_4^{3-} solutions was performed as follows. The samples (a disk ca. 10 mm in diameter) were placed in sterile 48-multiwell culture plates. Mouse fibroblasts cells (L929 cells) were seeded onto each sample within 1 cm^2 metallic rings at a density of 0.5×10^5 cells/disk in Eagle's MEM with 10% fetal calf serum. They were incubated at 37 °C in 5% CO_2 for 12 h for the cell adhesion test and for 48 h for the cell proliferation test. After washing with phosphate-buffered saline, the cells were detached using 0.05% trypsin in PBS, and the amount of cells adhered or proliferated on the substrates was counted with a hemocytometer.

Measurements. Wide-angle X-ray diffraction (WAXD) patterns were taken with a flat-plate camera mounted on a RIGAKU X-ray generator (ultraX18) emitting Ni-filtered Cu Kα radiation (40 kV, 200 mA) that was monochromated by a parabolic multilayer mirror and collimated by two pinholes (ϕ = 0.2 mm and 0.4 mm), in transmission geometry. Freeze-dried samples were used.

Fourier transformation infrared (FT-IR) spectra of the particle surface were recorded by the attenuated total reflection (ATR) method on a Perkin-Elmer Spectrum One FT-IR spectrometer after 64 scans (4 cm^{-1} resolution), over a range of 460−600 cm^{-1}. Freeze-dried samples were used.

The surface of the freeze-dried samples and cross-sections of the freeze-fractured samples were observed on a stage for scanning electron microscopic (SEM) observation with a HITACHI S-4100H SEM, after gold was sputtered onto the freeze-dried samples at a thickness of approximately 20 nm.

The morphology of the cells growing on the samples was studied by SEM. Immediately following the cell adhesion and proliferation tests, the samples were fixed with 3% GTA in PBS. After washing in PBS, the fixed samples were dehydrated in ethanol (25, 50, 90, 95, 99.5%) and 2-methyl-2-propanol. The dehydrated samples were then freeze-dried and fixed onto a stage for SEM observation. Before the SEM observation was performed, gold was sputtered onto the samples at a thickness of approximately 20 nm.

Results and Discussion

To prepare PVA hydrogels with an oriented structure, we attempted to chemically cross-link the network chains of the freeze−thawed PVA hydrogels under mechanical drawing. The procedure is schematically illustrated in Figure 1a. First, the aqueous solution of PVA (concentration; 10 wt %) was frozen at −20 °C and successively thawed at 4 °C, and this procedure was repeated 10 times. White and tough PVA hydrogels were formed as a result. Second, the freeze−thawed hydrogels were drawn 1, 2, 3, 4, and 5 times their original length. The hydrogels became somewhat brilliant and showed birefringence, which was confirmed by crossed-polarizing microscopy. The hydrogels were immersed into the acidic solution (HCl, pH = 2) just after being immersed in the GTA solution with concentrations of 0.6, 1.2, and 2.4 M corresponding-to-molar ratios of 5, 10, and 20 mol % to the hydroxyls of PVA, respectively. The hydrogels were then settled for 3 days, followed by repeated washing with pure water. In all cases, the shape of the hydrogel under the drawing conditions was fixed by the GTA treatment, even after they were released from the drawing force, strongly suggesting that the chemical cross-linking was effective for fixing the structural orientation. The swelling degree, q, defined as the weight ratio of the swollen gel to that of the dried gel, was more than 4.4, regardless of the preparation conditions (Table 1). The q values obtained were high and sufficient for calcium ions to interact with phosphate ions on/in the hydrogel matrix.

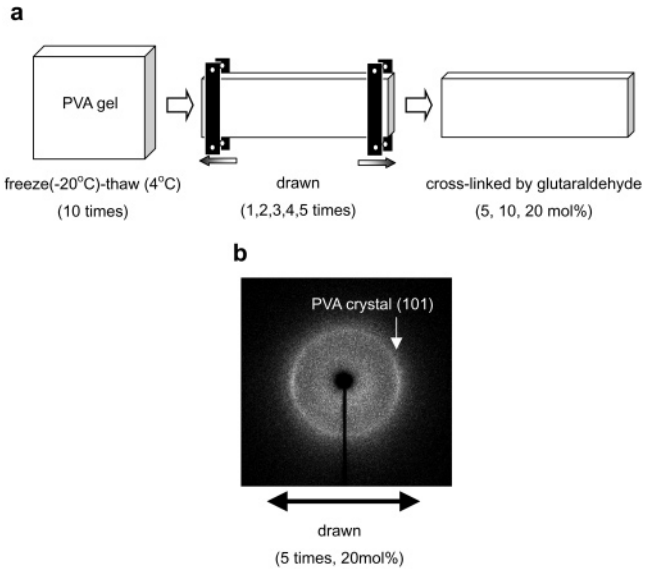

Figure 1. (a) Schematic illustration of the preparation procedure for the oriented hydrogels. (b) Wide-angle X-ray diffraction image of hydrogels drawn 5 times as long as their original length and chemically cross-linked with 20 mol % glutaraldehyde.

To confirm the orientation, wide-angle X-ray diffraction (WAXD) images of the drawn hydrogels were obtained. Figure 1b shows the WAXD image of a hydrogel drawn up to 5 times it original length and cross-linked with a 20 mol % GTA ratio. The diffraction arcs appeared at 2θ = 20.0° (θ: diffraction angle), corresponding to a spacing of 0.45 nm, and the arc center was located on the equatorial line corresponding to the drawing direction. Since this diffraction could be assigned to the (101) plane of the PVA crystal, corresponding to the spacing of the polymeric backbones,[11] these arcs indicated an orthogonal orientation of the

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Table 1. Preparation and Characteristics of PVA Hydrogels

drawing ratio[a] (mm/mm)	molar ratio of cross-linker[b] (mol %)	swelling ratio[c] (wt/wt)	orientation degree[d] (%)
1	5	7.4	not oriented
	10	5.6	not oriented
	20	4.4	not oriented
2	5	8.7	not oriented
	10	8.0	not oriented
	20	7.0	not oriented
3	5	8.0	not oriented
	10	6.4	not oriented
	20	6.2	not oriented
4	5	8.7	not oriented
	10	7.0	74
	20	5.1	75
5	5	8.1	70
	10	6.3	76
	20	5.1	79

[a] The length ratio of the hydrogels in the drawn state to that in the original state. [b] The molar ratio of GTA to the hydroxyls of PVA in the feed. [c] The weight ratio of the water-swollen hydrogels to the dried ones. [d] Calculated from the azimuthal scanning of a diffraction at $2\theta = 20°$.

crystallized PVA backbones, which formed the physical cross-linking domains, to the drawing direction. The orientation degree was roughly calculated as 79% from the results of the azimuthal scanning diagram of the (101) diffraction with the widely used equation[12] (360 − B)/360 × 100, where B is the sum of the full width at the half-maximum of two (101) diffraction arcs in the azimuthal scan. Hydrogels drawn up to 5 times their original length and cross-linked with 5 and 10 mol % GTA ratios and drawn up to 4 times their original length and cross-linked with 10 and 20 mol % GTA ratios also showed WAXD arcs on the equatorial lines. The orientation degrees of these hydrogels were calculated as 70−79% and increased with an increase in the drawing ratio and the GTA ratio, as shown in Table 1. Hydrogels prepared under a drawing ratio of 4 times and a low GTA ratio of 5 mol % showed a homogeneous Debye−Sherrer ring in contrast to the orientation. The orientation of hydrogels prepared under low drawing ratios of 1−3 times were also precluded by the WAXD study.

The chemically cross-linked hydrogels under the drawing conditions were alternatively immersed into aqueous solutions containing calcium chloride and sodium hydrogen phosphate. The alternate immersion treatment was repeated six times, which gave a sufficient amount of HAp for analyzing the mineralization behavior.[4−6] The WAXD diagram of the treated gels showed diffractions not only at $2\theta = 20.0°$ but also at $2\theta = 25.9°$, 28.1°, 31.8°, 32.2°, 32.9°, 39.8°, 46.7°, and 49.5°, which are characteristic of synthetic HAp crystals (hexagonal lattice; $a = 0.94$ nm, $b = 0.94$ nm, $c = 0.69$ nm),[13] although these diffraction peaks were broader than those of the general inorganic crystals.[13] Therefore, it can be confirmed that the HAp−PVA hydrogel hybrids were prepared from drawn hydrogels by the present alternate-immersion method, which was originally developed in a system using nondrawn hydrogels.[5] Figure 2 shows the amount of mineralized HAp per gel prepared under different reaction conditions. The amount of mineralized HAp was larger in the hydrogels with a higher q if the drawing ratio was the same, similarly with the case of nondrawn PVA hydrogels.[5] On the other hand, the amount of mineralized HAp showed a minimum when the drawing ratio was increased under the same GTA ratio. The drawing treatment reduced the thickness of the gel, which was successively punched into disks 10 mm in diameter, which reduced the volume of the samples. Therefore, a decrease in the amount of mineralized HAp per gel seems to be correct. Otherwise, the amount of mineralized HAp was increased beyond the drawing ratio of 4 times. This result may indicate that the orientation of the gel matrixes may have enhanced the HAp mineralization.

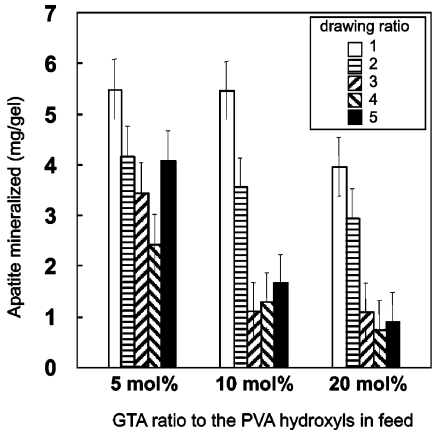

Figure 2. Amount of hydroxyapatite mineralized on/in hydrogels prepared under various drawing ratios and glutaraldehyde ratios to the hydroxyls.

In this case, the structure of the HAp crystal should be affected by the orientation of the hydrogels. Figure 3 shows the WAXD image of a hybrid of HAp with a hydrogel prepared under a drawing ratio of 5 times and a GTA ratio of 20 mol %. With keeping the PVA diffraction arcs on the equatorial line, distinct diffraction arcs appeared at $2\theta = 25.9°$ on the meridian lines, while the homogeneous diffraction ring appeared at $2\theta = 31.8°$. The former can be assigned to the (002) diffraction of the HAp crystal, and the latter can be assigned to the (211) diffraction. Weak diffractions at (102), (112), (300), (310), (222), and (213) in the HAp crystal also appeared, although the photographic resolution in this figure had low contrast and was difficult to distinguish. Although the (300) diffraction showed the six arcs located hexagonally, as shown in the illustration below the WAXD image in Figure 3, the others were Debye−Sherrer rings. Overall, both (002) and (300) diffractions corresponding to a one-dimensional arrangement in the crystal appeared as arcs, while the others corresponding to a three-dimensional arrangement appeared as rings. Since the diffraction arcs have too low of a contrast to confirm their positions, Figure 4 shows the results of the azimuthal scan of (002) and (300) diffractions from $\beta = -30°$ to 150° (β is the azimuthal angle and $\beta = 0°$ corresponds to the top on the meridian line). Although the (002) diffraction showed one distinct peak at around $\beta = 90°$, showing its position on the equatorial line, the (300) diffractions showed three peaks at around $\beta = 0°$, 60°, and 120°, confirming a hexagonal position. The

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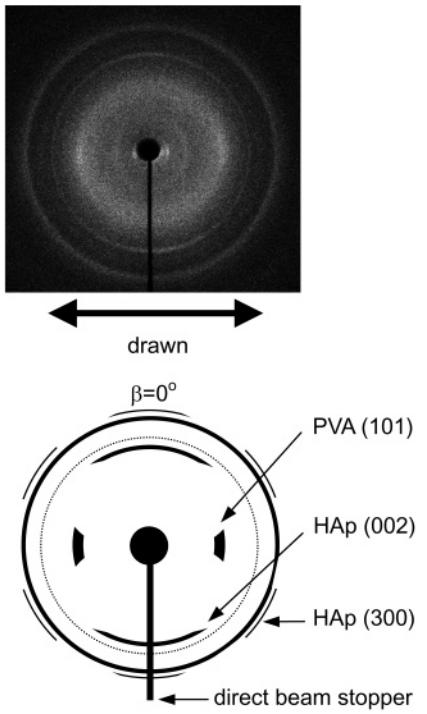

Figure 3. Wide-angle X-ray diffraction images of hydrogels drawn 5 times as long as their original length and chemically cross-linked with 20 mol % of glutaraldehyde and then alternatively immersed into aqueous solutions of calcium ion and phosphate ion. The noticeable diffraction and an explanation of the image are shown below.

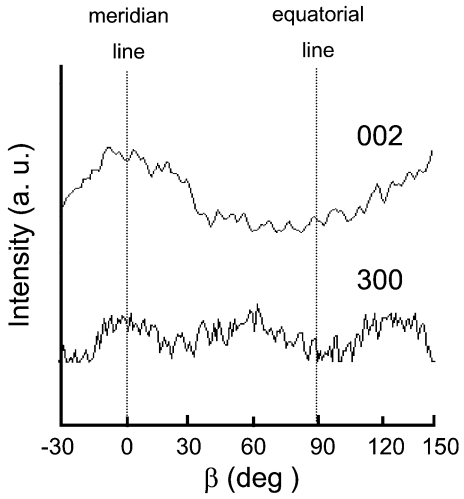

Figure 4. The azimuthal scanning diagram of (002) and (300) diffractions of the hydroxyapatite crystal. The original wide-angle X-ray diffraction image is shown in Figure 3.

orientation degrees calculated from the full width at the half-maximum of the peaks of the (002) and (300) diffractions were 85% and 75%, respectively, which were comparable to those of the hydrogel precursors. One possible illustration of the HAp crystal arrangement on the oriented PVA hydrogels is shown in Figure 5, where the *c*-axis is directed perpendicularly to the drawing direction of the hydrogels, the *a*-axis (equal to the *b*-axis in this hexagonal system) of the HAp crystal is directed hexagonally, and no three-dimensional arrangement is made. Such an orientation was also confirmed in the hybrid from the hydrogel prepared under a drawing ratio of 5 times and a GTA ratio of 10 mol %, but no

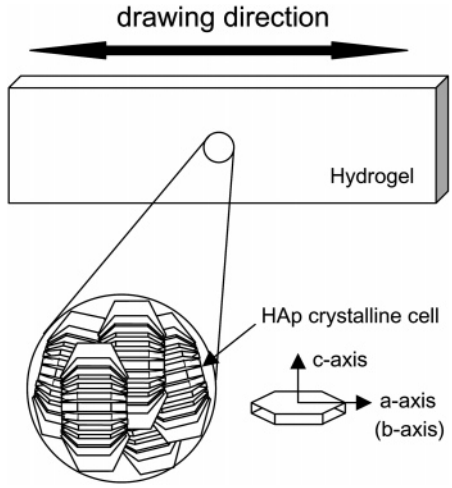

Figure 5. Schematic illustration of the oriented structure of the hydroxyapatite crystals on the hydrogels. The crystallized poly(vinyl alcohol) chains in the hydrogel are oriented vertically. The size of individual components such as the hydrogel and hydroxyapatite crystalline cells is arbitrary.

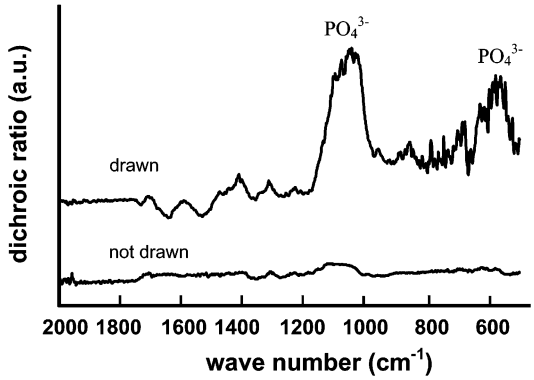

Figure 6. The dichroic ratio of the polarizing attenuated transmittance reflection/Fourier transformed-infrared (ATR/FT-IR) spectra of hydrogels drawn 5 times as long as their original length and then chemically cross-linked with 20 mol % glutaraldehyde.

orientation was confirmed in the hydrogels prepared under the other conditions. Thus, the highly oriented PVA network induced an orthogonal orientation of the HAp crystal to the drawing direction; in other words, a parallel orientation to the crystallized PVA chains. According to the report on the biomimetic mineralization of HAp on the drawn gelatin films doped with sodium polyacrylate,[14] the *c*-axis of the HAp crystal was oriented parallel to the cross-linking collagen triple helices, which were oriented parallel to the drawing direction. The HAp orientation should be determined by the orientation direction of the organized polymeric chains in the cross-linking domains.

To investigate the orientation at the molecular level, a polarizing FTIR/ATR study of the hybrid prepared under a drawing ratio of 5 times and a GTA ratio of 20 mol % was performed. Figure 6 shows the dichroic ratio of the IR transmittance recorded using a polarized laser parallel to that recorded using an orthogonally polarized laser. Although no peak appeared in the sample from the nondrawn hydrogel, a distinct dichroic ratio ap-

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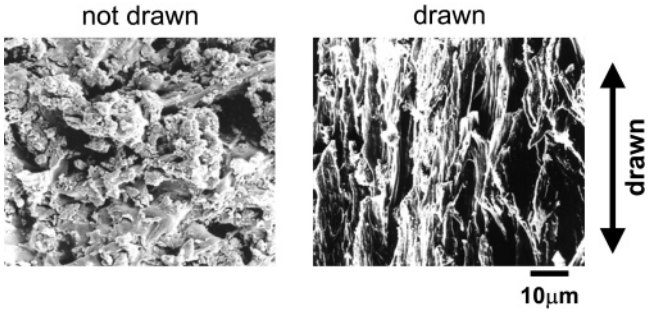

Figure 7. Scanning electron microscopic photographs of a freeze-fractured hybrid of hydroxyapatite with nondrawn hydrogels and with hydrogels drawn 5 times as long as its original length and then chemically cross-linked with 20 mol % glutaraldehyde.

peared at around 1050 and 590 cm^{-1}, which can be both assigned to the vibration of the phosphate ion.[15] This result suggests that the orientation of the hydrogel substrate for HAp mineralization affects the orientation of the phosphate ion. There are several reports on the anistropic mineralization of HAp on an organic substrate,[14−17] but none of them showed the orientation degree or the orientation of the phosphate ion.

To investigate the effects of the PVA orientation on HAp mineralization at the mesoscopic level, we took SEM photographs of the cross section of an oriented HAp−PVA hydrogel hybrid. Figure 7 shows the SEM micrographs of drawn and nondrawn samples from hydrogels prepared under a drawing ratio of 5 times and a GTA ratio of 20 mol %. One can confirm the homogeneous growth of HAp crystals on the micrograph of the nondrawn sample in contrast to the anisotropic morphology of the HAp crystals on the micrograph of the drawn sample. The drawing procedure was effective on changing the morphology of the HAp crystals at the mesoscopic level.

A previous report showed that HAp mineralized on the substrate had excellent cell compatibility.[8] The effects of the orientation of the HAp on cell adhesion and extension were investigated. Mouse fibroblast cells (L929) were seeded onto oriented and nonoriented hybrids of HAp−hydrogels prepared under a drawing ratio of 5 times and a GTA ratio of 20 mol %. The number of cells adhered onto the substrate is shown in Figure 8. In both oriented and nonoriented samples, the presence of HAp drastically increased the cell number. The hybrid orientation made the adhered cell number drop, which may be attributed to a decreased amount of mineralized HAp on the hydrogels.

The morphology of the extended cells was studied by SEM. Figure 9a shows SEM photographs of freeze-dried samples of cells extending onto the hybrid HAp with PVA hydrogels prepared under a drawing ratio of 5 times and a GTA ratio of 20 mol %. On the nonoriented substrate, L929 cells extended well and put out their pseudopodia in every direction, but the extension direction was random. With respect to the oriented substrate, L929 cells also extended well but were slightly fewer

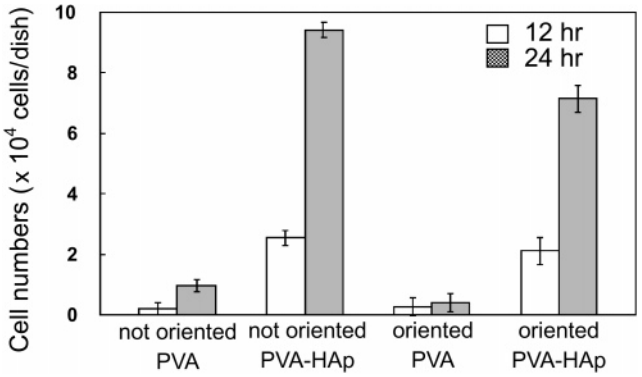

Figure 8. Number of cells adhered onto a hybrid of hydroxyapatite with a nondrawn hydrogel and with a hydrogel drawn 5 times as long as its original length and then chemically cross-linked with 20 mol % glutaraldehyde.

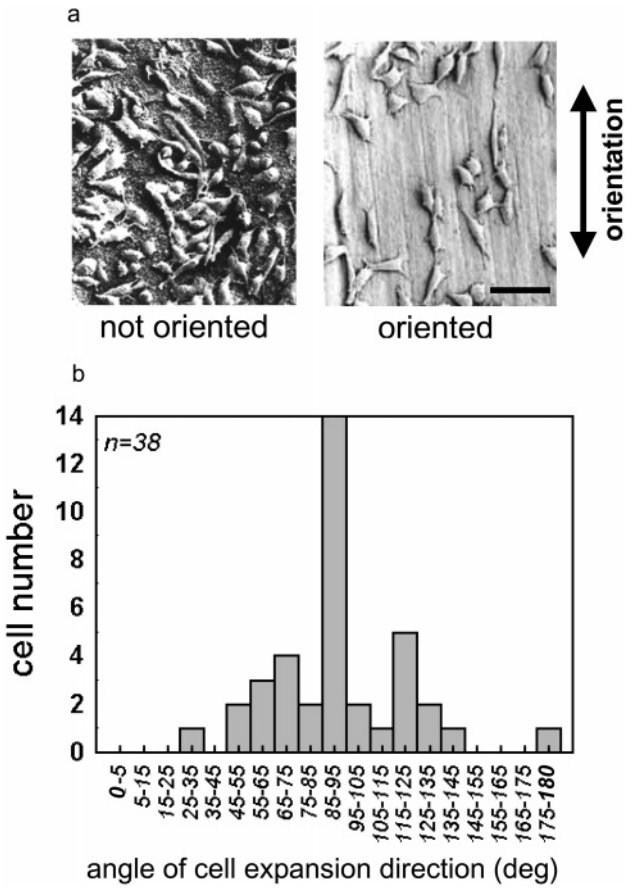

Figure 9. (a) Scanning electron microscopic photographs of mouse fibroblast cells extending onto a hybrid of hydroxyapatite with a nondrawn hydrogel and with a hydrogel drawn 5 times as long as its original length and then chemically cross-linked with 20 mol % glutaraldehyde. (b) The angle distribution of the longitudinal direction of the extended cells to the drawing direction of the hydrogels.

in number than in the nonoriented samples. Several cells also extended uniaxially, and the longitudinal axis of these cells was almost along the drawing direction. The angle distribution of the cell longitudinal direction to the hydrogel drawing direction was analyzed from the SEM images (Figure 9b). The median hydrogel drawing angle ranged from 85° to 95°, and almost all of the cells extended at angles ranging from 45 to 135°, showing that the cell extension was strongly influenced by the substrate drawing.

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Conclusions

We prepared HAp−PVA hybrids with a structural orientation by the procedure described below: (1) poly(vinyl alcohol) hydrogels were freeze−thawed 10 times; (2) the hydrogels were then uniaxially drawn up to 5 times their original length to give a structural orientation, which was fixed by successive chemical crosslinking by glutaraldehyde (20 mol %); (3) the oriented hydrogels were treated by repeated alternate immersion into solutions of phosphate ion and calcium ion, successfully mineralizing the hydroxyapatite (HAp) crystal whose *c*-axis was oriented perpendicularly to the surface of the hydrogel, which was confirmed by X-ray diffraction. In addition, polarized infrared spectroscopy confirmed the orientation of the phosphate ion. We then performed cell extension tests on the structurally oriented HAp−PVA hydrogel hybrids. Scanning electron microscopy confirmed that mouse fibroblasts (L929) extended on the oriented HAp-hydrogel hybrid along the drawing direction. This result shows the potential to control the direction of cell extension by substrate orientation, and also the possibility for reconstructing oriented bony tissue.

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