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Surface Hydrolysis of Fibrous Poly(ε-caprolactone) Scaffolds for Enhanced Osteoblast Adhesion and Proliferation

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Abstract: A procedure for the surface hydrolysis of an electrospun poly(ε-caprolactone) (PCL) fibrous scaffold was developed to enhance the adhesion and proliferation of osteoblasts. The surface hydrolysis of fibrous scaffolds was performed using NaOH treatment for the formation of carboxyl groups on the fiber surfaces. The hydrolysis process did not induce deformation of the fibers, and the fibers retained their diameter. The cell seeding density on the NaOH-treated PCL fibrous scaffolds was more pronounced than on the non-treated PCL fibers used as a control. The alkaline phosphatase activity, osteocalcin and a mineralization assay strongly supported that the surface-hydrolyzed PCL fibrous scaffolds provided more favorable environments for the proliferation and functions of osteoblasts compared to the non-treated PCL fibrous scaffolds use as a control.

Keywords: electrospinning, poly(ε-caprolactone), surface hydrolysis, osteoblast, tissue engineering.

Introduction

Electrospinning has been widely utilized for the production of fibers with a broad range of applications such as controlled release for antibiotics and heparin, gene therapy, and tissue engineering.[1-10] Among many advantages of fibrous structures, the well-defined, uniform dimension has shown many advantages, especially as tissue regenerating scaffolds, due to the a large surface-to-volume ratio as well as a interconnected pore structure with a high permeability.[1,11,12] Recently, electrospun biodegradable polymer fibers have been served as supporting matrices for seeding and growth of various cells.[11,13] In particular, fibers electrospun from poly(ε-caprolactone) (PCL) have shown the potential as scaffolds for tissue regeneration since they do not produce the serious problem caused by acidic degraded compounds, which is usually observed with polylactide (PLA) and poly(lactic-*co*-glycolic acid) (PLGA).[14-16] Besides, PCL has good mechanical properties as well as prolonged degradation time.[17]

To date, surface-treatment has been widely employed to supply the environments for better interactions with cells by increasing the surface hydrophilicity or wettability of PCL-based scaffolds.[18-20] Representative examples are the surface modification by grafting polymerization of acrylic acid on PCL films and further immobilization of collagen or gelatin, and the partial hydrolysis of PCL surfaces by NaOH treatments.[13,19,20] Surface hydrolysis is recognized as a simple, effective approach for generation of many kinds of degradable scaffolds, which can serve as good templates for a wide variety of cells such as chondrocyte, vascular endothelial and sooth muscle cells.[13,21,22]

In this work, we describe the effect of surface hydrolysis of PCL fibrous scaffolds on the osteoblast-scaffold interac-

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tion as well as osteoblast proliferation and functions. We are interested in knowing whether the partially hydrolyzed surfaces of PCL fibrous scaffolds can provide the surface environment favorable for osteoblast spreading and growth, compared with non-treated PCL fiber scaffolds. *In vitro* biological properties of this newly developed surface-engineered PCL fibrous scaffold were compared with those of conventional PCL fibrous scaffolds.

Experimental

Materials. Poly(ε-caprolactone) (PCL) with an average molecular weight of 80,000 g/mol was purchased from Aldrich Co. (Milwaukee, WI). Methylene chloride and sodium hydroxide (NaOH) were of the analytical grade and used as received.

Electrospinning of PCL. The PCL solution (10 wt%) in chloroform/ethanol (7 : 3 v/v) was transferred at a constant flow rate (5 μL/min) to a metal capillary connected to a high-voltage power supply. The electrospinning was performed in a chamber with constant temperature of 40 °C. Upon applying an electric voltage of 17 kV between the capillary and a metal collection plate, a fluid jet accelerated toward a grounded collector to form the fibers in the form of non-woven fabric. The fibers were dried in vacuo at room temperature for 2 days to remove the residual solvents.

Surface Hydrolysis of PCL Fibrous Scaffolds. PCL scaffolds were soaked in 1 N NaOH for 1 h at room temperature. Substrates were taken out and then rinsed extensively with distilled water until the supernatant pH became 7.4. The water contact angle was measured at room temperature by the sessile drop method using a goniometer (GonioStar200, SurfaceTech, Ltd) and Contact Angle Measurement V1.2 Software.

Osteoblast Isolation and Culture. Osteoblasts were isolated from the long bone of 4-weeks Sprague-Dawley rats by an enzymatic digestive method. The long bone was dissected, cut into small pieces, and rinsed in a sterilized phosphate-buffered saline (PBS) solution. The small pieces were incubated with 1% trypsin-EDTA for 10 min, followed by four sequential digestions with 0.06% collagenase at 37 °C for 45 min each. The digestions produced a suspension of cells with high proportion of osteoblasts. After centrifugation at 1,000 rpm for 10 min, each pellet was resuspended in 5 mL of Dulbecco's Modified Eagle's Medium (DMEM) supplimented 15% fetal bovine serum, and 1% antibiotics (penicillin 100 U/mL, streptomycin 0.1 mg/mL), 50 mM ascorbic acid, 0.1 μM dexamethasone and 10 mM β-glycerophosphate. The cells were seeded into 75-T tissue culture flasks, and allowed to grow in a controlled humidified incubator in the presence with 5% CO_2 at 37 °C. For experiments, cells were maintained to passage 3.

Surface Morphology of Osteoblast-cultured Fibrous Scaffolds. For morphology observation, fibrous scaffolds were taken out from the cell culture media and fixed with 2.5% glutaldehyde (Junsei Chemical Co., Ltd., Japan) at 4 °C for 1day. After fixation, the scaffolds were washed and dehydrated with EtOH for 10 min and freeze-dried. The morphology of the freeze-dried samples was observed using a Hitachi S2460N scanning electron microscope. The sample specimen was coated with Pt on a Cressington Scientific Instruments 108 auto sputter coater. The accelerating voltage was 5 kV.

The Effect of Surface Hydrolysis on Osteoblast Adhesion and Proliferation. NaOH-treated electrospun PCL scaffolds and non-treated PCL scaffolds were cut into a rectangular shape ($10 \times 10 \times 3$ mm^3). Before cell seeding, the scaffolds were sterilized by soaking in 70% EtOH for 30 min and washed extensively with PBS. Osteoblast of 2×10^5 cells/mL was seeded on each fibrous scaffold in a 24-well plate. Cells were propagated in Dulbecco's Modified Eagle's Medium (DMEM) supplimented 15% fetal bovine serum, and 1% antibiotics (penicillin 100 U/mL, streptomycin 0.1 mg/mL), 50 mM ascorbic acid, 0.1 μM dexamethasone and 10 mM β-glycerophosphate at 37 °C in a humidified incubator with 5% CO_2. Cell proliferation on scaffolds was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. After 1, 7, 14, 21, and 28 days, 100 μL of a 5 mg/mL MTT solution in PBS was added to each well, and the plate was incubated for 4 h at 37 °C, allowing viable cells in scaffolds to reduce the MTT into purple formazan crystal. After incubation period, the formazan crystal was dissolved by adding 1 mL of dimethyl sulfoxide (DMSO) and equilibration for 3 h. The absorbance of solutions in individual wells was measured at 570 nm by a microplate reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA).

Alkaline Phosphatase Activity. The scaffolds were washed with PBS, homogenized with 2 mL lysis buffer solution (0.02% Triton X-100, Sigma) for 2 min. Grinded scaffolds were then purified by centrifugation at 14,000 rpm for 15 min. Supernatants were collected after 1, 7, 14, 21, and 28 days (n = 3), frozen at -20 °C and then thawed prior to analysis. The enzyme reaction was set up by mixing 6 μL of the sample with 54 μL of lysis buffer (0.02% Triton X-100/saline) containing 100 μL of 1 M Tris-HCl (pH 9.0), 20 μL of 5 mM $MgCl_2$, and 20 μL of 5 mM *para*-nitrophenyl-2-phosphate (PNPP). The solution was incubated at 37 °C for 30 min, and the reaction was then stopped by an aqueous NaOH solution (1 N). The level of *para*-nitrophenol production was measured by monitoring the light absorbance of the solution at 405 nm using a microplate reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA). Results are shown in nmole/protein/min.

Osteocalcin Assay. Osteocalcin production from osteoblast-scaffold constructs was assessed using a commercial enzyme immunoassay kit (Biomedical Technologies Inc., Stougton, MA, USA). A 2 mL of media was used for the

extraction of osteocalcin, and 1 mL of the supernatant was subjected to assay. Results are presented in ng/mL.

Calcium Assay. To quantify calcium deposited in the fibrous scaffolds, sample scaffolds were washed with PBS and homogenized with 0.6 N HCl. Calcium was extracted by shaking for 4 h at 4 °C. After centrifugation at 1,000 g for 20 min, the supernatant was subjected to assay to determine the calcium content. After addition of cresolphthalein complexone (Sigma Co.) into the supernatant and equilibration for 5 min, the calcium concentration was determined by measuring absorbance at 570 nm using a microplate reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA). The data were normalized to a standard curve obtained with calcium concentrations in the range of 10-120 μg/mL.

von Kossa Staining. Scaffolds were stained in a 5% silver nitrate ($AgNO_3$) solution for 60 min. Samples were rinsed with distilled water and then soaked in a 5% sodium thiosulfate solution for 5 min at room temperature. Stained scaffolds were washed twice with distilled water. The scaffolds were observed by light microscope (CK40-F200, Olympus Optical Co., Tokyo, Japan).

Results and Discussion

Surface Morphology of Osteoblast-Cultured Scaffolds. Electrospun PCL scaffolds exhibited a non-woven fibrous structure, as shown in Figure 1. The diameter of fibers in three-dimensional scaffolds is in the range of 1-3.5 μm, and the average diameter was found to be 2 ± 0.1 μm. The scaffolds consist of the randomly oriented non-woven fibers and have the interconnected uniform pore structures. The surface of the fibers is generally regular and does not have the significant defects. The surface hydrolysis of fibrous scaffolds did not induce the changes in morphology and size, and the smooth surface of fibers was maintained. This observation supports the selective hydrolysis of the fiber surfaces. The hydrophilicity and wettability of the PCL fiber surface can be changed by NaOH treatments. To estimate this variation, we measured the water contact angle on the surface-hydrolyzed PCL fibrous scaffold and non-treated PCL scaffolds. Contact angles were $44 \pm 7°$ and $102 \pm 3°$ for the surface-hydrolyzed scaffold and the non-treated scaffold, respectively. This indicates that NaOH-treatment made the PCL fiber surface more hydrophilic or polar, compared with that of the non-treated PCL scaffold. Figure 1 shows the effect of surface hydrolysis of PCL fibrous scaffolds on osteoblast seeding and proliferation. At 1 day, the effect of surface hydrolysis on osteoblast adhesion is negligible. It is interesting to note that, after 3-day culture, the surface of NaOH-treated scaffolds was covered about 75% with cell multilayers, whereas the cell adhesion and spreading are not found effective on non-treated fibrous scaffolds. At 5 and 7 days, the surface-hydrolyzed scaffolds have the multilayers of osteoblasts which cover almost

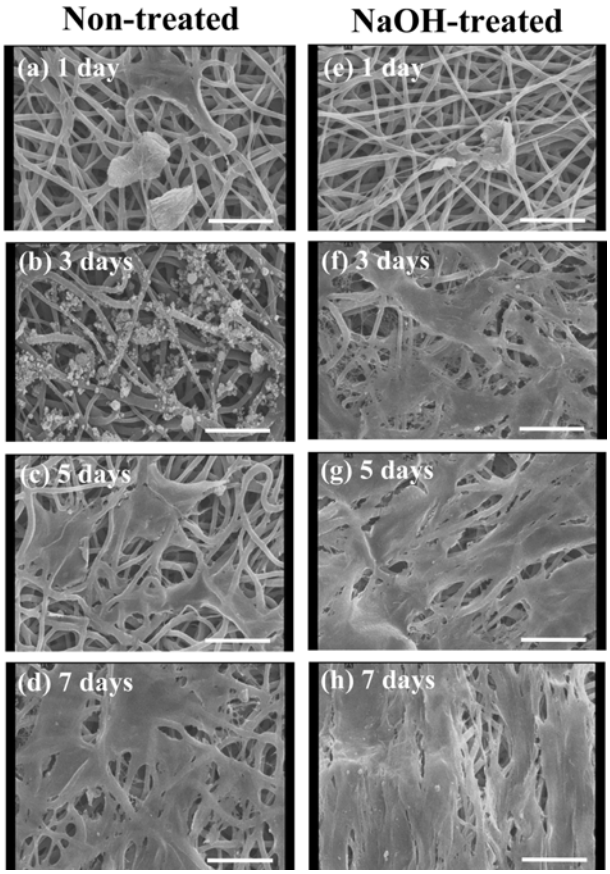

Figure 1. SEM micrographs of osteoblast-cultured electrospun PCL fibrous scaffolds. Scale bars represent 30 μm.

completely the scaffold surfaces. At the same time period, the cells began to spread and grow on the non-treated control fibrous scaffold, but not to a comparable extent to the NaOH-treated one. This positive effect of surface hydrolysis on increased cell densities can be understood in terms of the enhanced adsorption of serum proteins. Most mammalian cells such as osteoblast are anchorage-dependent cells which adhere to the surfaces via the interaction with pre-adsorbed proteins such as fibronectin and vitronectin. It was reported that the anionic groups such as sulfonic and phosphonic acids contributed to the enhanced pre-adsorption of serum proteins.[22-24] For this reason, the generation of carboxylic acid groups on the fiber surface by NaOH-treatment is the key in inducing the effective osteoblast adhesion.

The Effect of Surface Hydrolysis on Osteoblast Proliferation. Osteoblast proliferation on surface-hydrolyzed fibrous scaffolds was compared with that on non-treated scaffolds. As shown in Figure 2, gradual osteoblast growth was observed on both the hydrolyzed scaffold surface and non-treated one. It is noted that the initial adhesion number of cells on hydrolyzed scaffold is more than that on non-treated scaffolds. As the incubation time increased, cell number gradually increased after that period. It is notewor-

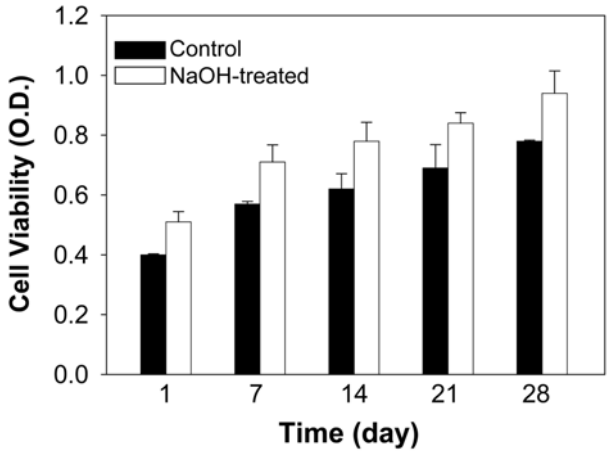

Figure 2. Comparison of cell viability cultured on non-treated PCL scaffolds and NaOH-treated PCL scaffolds (n = 3).

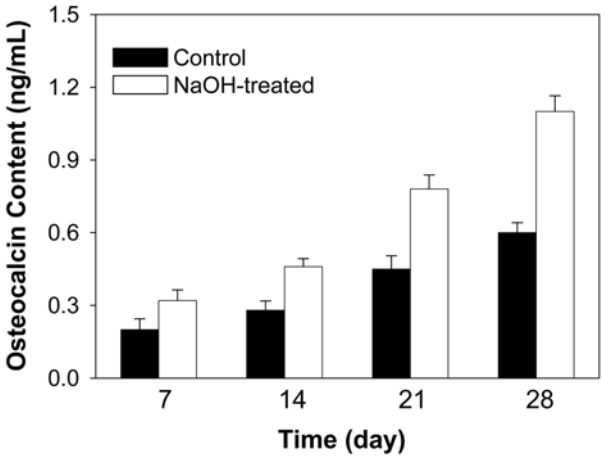

Figure 4. Osteocalcin contents of osteoblast-cultured non-treated PCL scaffolds and NaOH-treated PCL scaffolds (n = 3).

thy that the osteoblast numbers are greater on NaOH-treated scaffolds compared to non-treated scaffolds at a broad culture period of 1-28 days. This tendency for cell proliferation is consistent with the morphological observation described in Figure 1. This suggests that the enhanced surface wettability and hydrophilicity of the surface-hydrolyzed fibrous scaffolds are main contributors to induce the effective cell-scaffold interaction as well as cell growth.

Alkaline Phosphatase Activity. The osteoblastic function on fibrous scaffolds was evaluated by measuring the activity of alkaline phosphatase, a membrane enzyme routinely used *in vitro* experiments as a relative marker of osteoblastic growth and functions.[25] As shown in Figure 3, the osteoblast growth on NaOH-treated hybrid scaffolds showed a more significant enhancement than that on control scaffolds, as judged by ALP activity, during the 4-week culture period. This indicates that surface-hydrolyzed fibrous scaffolds could provide the favorable environments for osteoblast functions.

Osteocalcin Assay. The content of osteocalcin, a specific marker for osteoblast, is a good measure to decide whether the scaffolds can offer the environments favorable for osteoblast growth and functions.[26] Figure 4 shows the osteocalcin content on cell-cultured non-treated and NaOH-treated PCL fibrous scaffolds, respectively. During the culture period of 1-4 weeks, the osteocalcin content on non-treated scaffolds showed the slight increase from 0.25 to 0.58 ng/mL. Interestingly, osteoblast-cultured surface-hydrolyzed scaffolds showed the greater content of osteocalcin in the range of 0.37-1.18 ng/mL at the same culture period.

Calcium Assay and von Kossa Staining. The effect of surface hydrolysis on the occurrence of mineralization in the osteoblast-cultured fibrous scaffolds was estimated by quantification of calcium contents and von Kossa staining

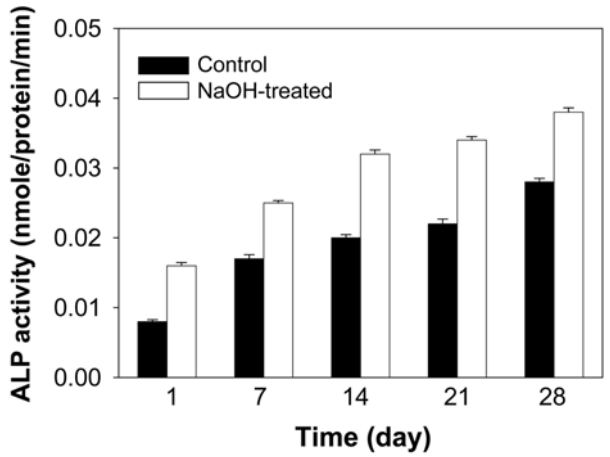

Figure 3. Alkaline phosphatase activity of osteoblast-cultured non-treated PCL scaffolds and NaOH-treated PCL scaffolds (n = 3).

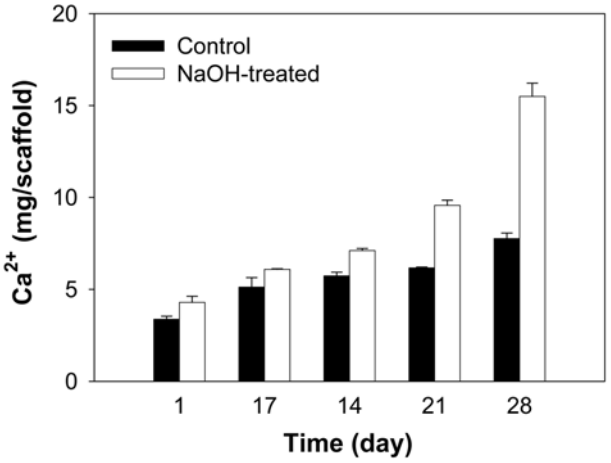

Figure 5. Calcium contents on osteoblast-cultured non-treated PCL scaffolds and NaOH-treated PCL scaffolds (n = 3).

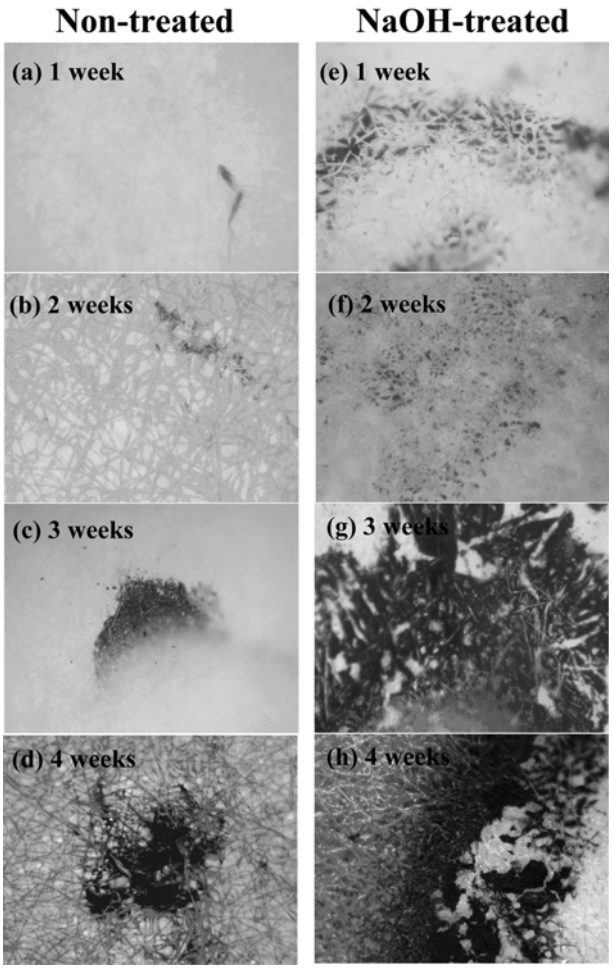

Figure 6. von Kossa staining of osteoblast-cultured non-treated PCL scaffolds and NaOH-treated PCL scaffolds.

to identify calcium depositions. Figure 5 shows the content of calcium deposition generated by osteoblast-cultured fibrous scaffolds over the 4-week culture period. The calcium deposition on non-treated and NaOH-treated PCL fibrous scaffolds gradually increased during the culture period. The calcium content on cell-cultured hydrolyzed scaffolds was greater than that on non-treated scaffolds. Figure 6 shows the stained image of osteoblast-cultured NaOH-treated and non-treated PCL fibrous scaffolds, respectively. At 1-2 weeks, the calcium deposition (shown as black spots) by osteoblast-induced mineralization on surface-hydrolyzed scaffolds was obviously occurred even at a short period of the cell culture time. On the other hand, the calcium deposition was not effective on non-treated scaffolds. It is interesting to note that, after 3 weeks, the block spot covers the whole area of the osteoblast-cultured surface-hydrolyzed fibrous scaffolds, whereas the substantial calcium deposition was found on non-treated scaffolds only after 3 weeks. Thus, this indicates that osteoblasts express bone mineral-forming functions more actively on surfaces of hydrolyzed PCL fibrous scaffolds than non-treated scaffolds.

Conclusions

This work describes a fabrication method of surface-hydrolyzed PCL fibrous scaffolds and their potentials as a bone-regenerative material. SEM analyses showed that osteoblasts were attached and proliferated on NaOH-treated PCL fibrous scaffolds more effectively than on the control non-treated PCL scaffolds. *In vitro* biological evaluation for osteoblast functions (bone-forming properties) strongly supported that the surface-hydrolysis of PCL fibers could provide the three-dimensional scaffolds with favorable environments for cell-biomaterial interactions as well as cell adhesion/proliferation. These findings suggest the potential of surface-hydrolyzed PCL fibers as scaffolds for bone tissue regeneration.

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