Initial Characterization of a Fibroblast-loaded Porous Elastin Film Reconstituted by a Novel Crosslinker, Dode-DSP

Kikuo Komori,*[1] Sumi Takamiya,[1] Nobuhiko Kojima,[1] Osamu Kuwazuru,[1] Nobuhiro Yoshikawa,[1] Katsuko Furukawa,[2] Kenichi Miyamoto,[3] and Yasuyuki Sakai[1]

[1]*Institute of Industrial Science, The University of Tokyo, Komaba, Meguro-ku, Tokyo 153-8505*

[2]*Department of Mechanical Engineering, Faculty of Engineering, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-8656*

[3]*Department of Chemistry for Materials, Faculty of Engineering and Graduate School of Engineering, Mie University, Kurimamachiya-cho, Tsu 514-8507*

(Received June 8, 2009; CL-090546; E-mail: kkomori@iis.u-tokyo.ac.jp)

A reconstituted porous elastin film prepared by a novel cross-linker, Dode-DSP, was examined for its feasibility as a biocompatible and elastic cellular scaffold. Although the mechanical properties of the film alone drastically deteriorated in physical conditions due to degradation, loading of fibroblasts retarded the deterioration in terms of distensibility and *E* value (Young's modulus).

An elastin-based cellular scaffold is useful for fabricating elastic tissues in vitro. Elastin is a key native constitute of elastic tissues[1] and has an important role in maintenance of tissue elasticity. It is believed that lysine residues in elastin molecules are involved in a crosslinking bond via desmosine and/or isodesmosine, resulting in exhibition of the elasticity in vivo. However, reconstitution of the scaffold using native elastin is almost impossible due to its insolubility resulting from a highly rigid crosslinked structure. Because of this situation, some research groups focused on using soluble elastin and/or elastin-like peptide due to their high solubility and developed their chemically crosslinked gels and films.[2] In most of them, however, the crosslinking bond was involved in not only amino but also carboxylic groups in those elastin molecules. Namely, the structure of those gels and films was markedly different from that of native elastin. In contrast, glutaraldehyde, which can react with amino groups, is widely used as a crosslinker, but it is toxic to living organisms including cells if it remains.[3]

Recently, Miyamoto and co-workers have synthesized a novel hydrophilic crosslinker, Dode-DSP (see Supporting Information (SI) Figure S1).[4,5] Dode-DSP can preferentially react with amino groups, so that it can link a protein and another via amide bonding. Based on this, they have been successful in formation of a chemically crosslinked elastin film and investigated its structure and mechanical properties.[6] However, they still have not evaluated the cell-loaded elastin film for long incubation periods. Herein, we prepared elastin film loaded with mouse fibroblast cell line, NIH3T3. Fibroblast cells play a significant role in tissue regeneration because of secretion and arrangement of a precursor elastin, tropoelastin,[7] in addition to growth factors, cytokines, and extracellular matrix molecules. We examined the cell stability and its distribution in the film after sufficient incubation. Additionally, we determined a value of Young's modulus for the cell-loaded elastin film as an index of mechanical properties.

The water-soluble elastin was obtained according to a previous report.[8] An aqueous solution containing 20 wt % water-soluble elastin and 24 mM Dode-DSP was injected into an intervening gap between glass by use of a 1-mm silicon sheet and then heated at 80 °C for 5 h, resulting in formation of a porous elastin film due to phase separation. The elastin film was immersed into a 70% aqueous EtOH solution for 3 h for sterilization and then transferred to Dulbecco's modified eagle medium (DMEM, Kojin Bio) containing 10% calf serum (Gibco), 20 mM hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 100 units mL^{-1} penicillin (Wako), 100 μg mL^{-1} streptomycin (Wako), and 0.25 μg mL^{-1} amphotericin B (Sigma) for 72 h because of removal of EtOH and unbound species. Herein, film thickness was determined to be ca. 350 μm. The elastin film was further transferred to 0.01 M aqueous HCl solution containing 10% collagen (Nitta Gelatin) at 37 °C for 30 min, resulting in physical adsorption of collagen in the film. After these treatments, NIH3T3 cells (Riken Cell Bank) were seeded to one side of the elastin film at a cell density of 7.0×10^4 cells cm^{-2} and maintained for 4 h in an incubator (37 °C and 5% CO_2), and then the same number of NIH3T3 cells was seeded to another side of the film, followed by keeping in the incubator.

To evaluate an activity of the cells loaded on the elastin film, the amount of glucose consumption in DMEM was determined by a glucose analyzer (Beckman). The number of cells was estimated from an extract intracellular DNA concentration using 4′,6-diaminodino-2-phenylindol fluorometry.[9] Distribution of cells loaded on the elastin film was observed with a confocal laser scanning microscope (CLSM, Olympus) after cytoplasmic stain with calcein-AM.

Uniaxial tension tests were performed in a 1.5 mM aqueous NaCl solution at 35 °C by means of a tensile testing machine consisting of a load cell (Showasokuki) that allowed measurement of vertical force in the range of 0.025–10 N for loading velocities at 5 mm min^{-1} and an autograph as a measures displacement (Shimadzu). As the elastin film was clipped at both ends of 1×2 mm^2 in area, net length of the film was 12 mm.

First, we tried to culture the cell-loaded film to proliferate NIH3T3 cells as much as possible. The glucose consumption by the cells immediately increased up to on Day 6 and then leveled off (Figure 1). This result indicates that increase in the glucose consumption is based on cell proliferation, whereas the constant region is based on growth arrest of the cells. As the glucose consumption reached equilibrium, we further determined the actual number of cells loaded to the elastin film incubated for 7 days. As a result, NIH3T3 cells were ca. $5.7 \pm 0.6 \times 10^6$ cells cm^{-3} (mean ± SE, $n = 5$) on a 350-μm thick film. This

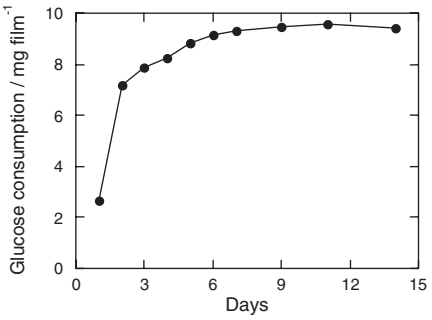

Figure 1. Changes in glucose consumption of NIH3T3 cells loaded to the elastin film in DMEM containing 5 g L^{-1} glucose.

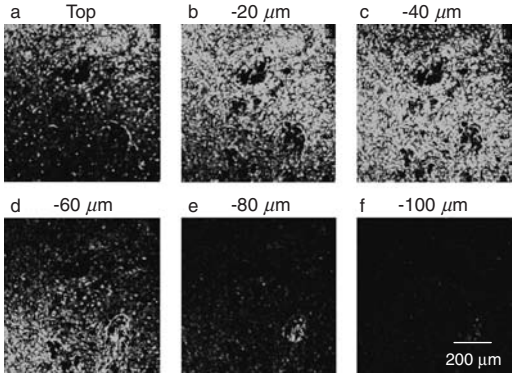

Figure 2. Fluorescent microphotographs of NIH3T3 cells, stained with calcein-AM, in the elastin film. The pictures were taken from the surface to 100 μm deep (a)–(f).

means that initially seeded cells to both sides of the film proliferated well up to 1000 times.

Using CLSM, we next confirmed distribution of the cells loaded on the elastin film on Day 7 (Figure 2). As a result, the cells were densely populated in the film up to a depth of 60 μm from both surfaces of the film, whereas few cells proliferated in further deeper regions. Cells might hardly intrude into such deeper region of the film, because the pore size of the porous elastin films prepared in this study was less than 20 μm (see SI, Figure S2).[5] If cells are seeded to both sides of the film of 120 μm in thickness or smaller, whole elastin film is expected to be filled with cells.

Next, in order to evaluate the mechanical properties of the cell-loaded elastin film, uniaxial tension tests were performed. Herein, we compared the cell-loaded elastin film with the cell-free film after 14 days of incubation. We also employed a cell-free elastin film after 3 days of incubation as a comparative experiment. As a result, strain values of those films increased linearly to stress values up to the maximum tensile strength, and then the stress values immediately reduced to zero due to shear rupture of the films. Based on this, the value of Young's modulus, E, for each elastin film was defined to be σ/ε, where σ is stress and ε is strain, respectively. The low E value shows it to be deformable.

Table 1 shows the E values, the rupture tensile strength values, and the distensibilities (strain at rupture) for each film. Each value is the average observed from three independently prepared films. Unfortunately, these values of the cell-loaded and the cell-free elastin films after 14 days of incubation were smaller than

Table 1. Mechanical property comparison of the cell-loaded elastin film with the cell-free one ($n = 3$)

	E value /kPa	Rupture tensile strength /kPa	Distensibility (Strain at rupture)
Cell-free film (3 days)	61 ± 3	27.5 ± 0.1	0.45 ± 0.07
Cell-free film (14 days)	30 ± 2	9.0 ± 0.1	0.30 ± 0.05
Cell-loaded film (14 days)	36 ± 3	13.7 ± 0.1	0.38 ± 0.04

those of the cell-free film after 3 days of incubation. The decrease in those values may be explained in that amide bonding between Dode-DSP and the elastin molecule might undergo hydrolysis, resulting in crosslink cleavage. In contrast, comparing the cell-loaded elastin film with the cell-free after 14 days of incubation, those three values of the former were larger than those of the latter. This result might indicate that secretions from NIH3T3 cells facilitated a crosslinking reaction among elastin molecules. For instance, fibroblast cells are known to secrete lysyl oxidase, which catalyzes a conversion of an ε amino group of lysine residue to a reactive aldehyde group.[10] Based on this, the aldehyde group might chemically bind to the other lysine residue in elastin. In addition, it might be also considered that the cells formed tight junctions based on their high-density population in the adjacent surface of the film as mentioned above, resulting in induction of increase in the film strength. Although the details are yet unclear, loading of fibroblasts certainly enhanced the mechanical properties of the reconstituted elastin film. Thus, the elastin film prepared in this study is likely to be employed as an elastic scaffold.

In future work, to fill the elastin film with the cells entirely, the film thickness needs to be reduced as well as enlarge the pore size of the film, so that cells can easily intrude to deeper sites. Additionally, to elucidate loss of the film strength based on immersion in the culture medium, it might necessary to measure changes in the film structure.

References and Notes

1 W. F. Daamen, J. H. Veerkamp, J. C. van Hest, T. H. van Kuppevelt, *Biomaterials* **2007**, *28*, 4378.
2 P. Buijtenhuijs, L. Buttafoco, A. A. Poot, W. F. Daamen, T. H. van Kuppevelt, P. J. Dijkstra, R. A. I. de Vos, L. M. Th. Sterk, B. R. H. Geelkerken, J. Feijen, I. Vermes, *Biotechnol. Appl. Biochem.* **2004**, *39*, 141; D. T. Simionescu, Q. Lu, Y. Song, J. S. Lee, T. N. Rosenbalm, C. Kelley, N. R. Vyavahare, *Biomaterilas* **2006**, *27*, 702.
3 L. H. H. Olde Damink, P. J. Dijkstra, M. J. A. van Luyn, P. B. van Wachem, P. Nieuwenhuis, J. Feijen, *J. Mater. Sci.: Mater. Med.* **1995**, *6*, 460.
4 K. Miyamoto, M. Tokita, T. Komai, *Protein Pept. Lett.* **2001**, *8*, 231.
5 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.
6 E. Kitazono, H. Kaneko, T. Miyoshi, K. Miyamoto, *J. Synth. Org. Chem. Jpn.* **2004**, *62*, 514.
7 L. Debelle, A. M. Tamburro, *Int. J. Biochem. Cell Biol.* **1999**, *31*, 261.
8 L. B. Sandberg, N. Weissman, D. W. Smith, *Biochemistry* **1969**, *8*, 2940.
9 C. F. Brunk, K. C. Jones, T. W. James, *Anal. Biochem.* **1979**, *92*, 497.
10 a) H. M. Kagan, P. C. Trackman, *Am. J. Respir. Cell Mol. Biol.* **1991**, *5*, 206. b) L. I. Smith-Mungo, H. M. Kagan, *Matrix Biol.* **1998**, *16*, 387.

Published on the web (Advance View) August 1, 2009; doi:10.1246/cl.2009.878