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Formation of Patterned Cellular Chips by Ion-beam Irradiation into Biodegradable Polymer

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We previously reported that thin films and self-assembly cellular sheets were obtained by ion-beam irradiation into biodegradable polymer. He^+ ion-beam irradiation improved cell attachment properties simultaneously. The aim of the study is to obtain micro-patterned cellular chip by ion-beam technology. Poly-$_L$-lactic acid (PLLA) sheets were used as substrates. He^+ ion-beam irradiation was performed at an energy of 150 keV with a fluence of 1×10^{15} ions/cm^2 using micro-patterned metallic masks. Bovine aorta endothelial cell attachment on micro-patterned ion-beam irradiated PLLA surfaces was improved dramatically. This surface with micro-patterned cells attached was exfoliated spontaneously from the substrate in a water solution. There were several cells on the micro-patterned cellular chips, and these chips were easily moved using a micromanipulator in the water solution. The number of cells on the micro-pattered cellular chips can also be controlled by ion-beam irradiation using freely designed micro-patterned masks of wanted. Two-dimensional patterned cell surfaces will be obtained using this technique. These techniques were useful for providing novel devices for developing cell technology and clinical medicine.
Key words: scaffold, tissue engineering, regenerative medicine, patterned cell culture

1. INTRODUCTION

About a century has passed since tissue-culture technology was born. Tissue culture technology had been developed through explant tissue cultures and organ cultures before the 1950s. However, cell cultures expanded after the 1960s due to the spread of useful cell culture media, cell-dispersing enzymes, and disposable plastic culture utensils. Instead of merely culturing cells on the flat bottom surface of glass or plastic culture vessels and growing the two-dimensional monolayer cells, studies appeared after the late 1960s that investigated inducing cell functions (growth, differentiation, and tissue reconstruction) by culturing cells on the materials while devising the structure and components [1].

In recent years, the shortage of organ donors and tissues has produced a significant need for organ and tissue replacements by new biological substitutes regenerated from tissue-specific cells and natural or synthetic polymeric matrices. These polymers play a crucial role in tissue engineering as scaffolds to support cells and as carriers of growth factors to allow their controlled release. The polymers applied for these purposes should meet certain requirements [2, 3]. Furthermore, micro-patterned immobilization of various biological molecules on substrates has been performed to assay cell functionality. The micro-patterned surface, which had good adhesive properties for cells or DNA, is expected to provide *in vitro* co-culture systems without any coatings of biological molecules [4].

Ion implantation is a unique method of modifying surface structures and properties of materials. Most research efforts in the field of ion-beam irradiation have concentrated on inorganic materials such as metals, ceramics and semiconductors. In recent years, ion-beam irradiation into polymers was investigated with ion-beam irradiation being applied to modify polymer surfaces to improve their compatibility with blood and tissue [5-15]. However, biomedical polymers using local energy deposition induced by ion-beam irradiation have not been investigated for use in the medical field. Our previous study reported that ion-beam irradiation into a biodegradable polymer produced a thin-film, self-assembled cellular sheet or spheroid, which exfoliated spontaneously from the substrate in a water solution [16].

PLA is a biodegradable polymer that hydrolyzes into a low molecule when implanted into a body. The hydrolysis-generated reaction products are non-toxic to the body since they are broken down into carbon dioxide and water by the body's metabolic cycle. The family of aliphatic polymers derived from lactide stereomers and other lactones, especially glycolide and ε-caprolactone, are currently a source of biodegradable materials for temporary therapeutic applications, namely suture materials, bone fracture internal fixation devices in surgery, and drug delivery systems in pharmacology.

Our previous study reported that He^+ ion-beam irradiation formed a thin film at an energy of 150 keV with a fluence of 1×10^{15} ions/cm^2 and a film thickness of 1.2 μm

after dipping in a phosphate-buffered saline solution (PBS(-)). The optimal irradiation fluence for forming a thin film was chosen by following reasons. Ion-beam irradiation with fluences of 1×10^{13} ions/cm^2 and 1×10^{16} ions/cm^2 did not form films. The 1×10^{13} ions/cm^2-ion-beam irradiation with slight radiation damage did not form a thin film because this damage may not bring bond scission. On the other hand, 1×10^{16} ions/cm^2-ion-beam irradiation also did not form a thin film because the carbonization was induced.

The 1.2 μm-thickness of the sheet was comparable to the peak value of energy deposition estimated by TRIM code (IBM, Co., USA) including the theory of Linhard et al. (LSS) [17].

The purpose of this work is to form micro-patterned surfaces and micro-patterned cellular chips by ion-beam irradiation into PLLA. Surface properties of the micro-patterned surface and chip were also investigated by means of surface-texture measuring instrument (STMI), scanning electronic microscope (SEM) and optical microscope.

2. MATERIALS AND METHODS

The substrates used were Poly-$_L$-lactic acid (PLLA) sheets (LACTY; SHIMADZU, Co., Kyoto, Japan) fabricated on 3 cm×3 cm. The atomic density of PLLA was 1.27 g/cm^3, and the thickness of the sheet was 200 μm. Figure 1 presents the formula for PLA. PLA incorporated a mirror image isomer such as PLLA or PDLA. In this report, we selected and used PLLA sheets for the experiments.

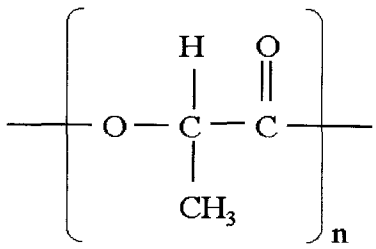

Fig. 1. Constitutional formula of Poly-lactic acid

We performed He^+ ion-beam irradiation at an energy of 150 keV with a fluence of 1×10^{15} ions/cm^2 at room temperature using the RIKEN 200 kV Low Current Implanter. The beam-current density was kept below 0.1 μA/cm^2 to prevent heating of the specimen. The target chamber pressure was maintained at a base pressure of 10^{-4} Pa during ion-beam irradiation. Micro-patterned ion-beam irradiation onto the surface was performed by putting a micro-patterned stainless mask on sample surfaces.

Micro-patterned irradiated surfaces were studied using a scanning electronic microscope (SEM, JSM-6330F : JEOL, Japan) after the sample surface was coated by Au^+ (30 nm) with a quick coater (SC-701: SANYU DENSI, Japan).

The surface morphology formed by ion-beam irradiation was measured using a surface-texture measuring instrument (STMI: SURFCOM 1400D, ACCRETECH, Japan). Post-exfoliation depression depth was measured using means of STMI.

We performed cell cultures on ion-beam irradiated surfaces and micro-patterned irradiated surfaces before exfoliation from the base material. Bovine aorta endothelial cells (BAECs) and, Mouse fibroblasts (L929) were used. BAECs were obtained from Sanko-jyunyaku (Japan) and L929 was acquired from the RIKEN Cell Bank (Tukuba, Japan). BAECs and L929 were suspended in a culture medium (RPMI 1640; Nissui Pharm., Co., Japan) supplemented with 10% fetal bovine serum (FBS; CCS SO7200, Sanko-Junyaku Co., Japan). We initially seeded cells at 2×10^5 cells/dish then incubated them at 37 °C in a humidified atmosphere with 5% CO_2. The extent of cell attachment and spreading was determined visually with an optical microscope equipped with phase contrast objectives and a CCD camera (IX-70; Olympus Co., Tokyo, Japan).

Two-centimeter square ion-beam irradiated PLLA specimens were prepared in order to estimate cell growth. The number of spread cells was counted on the photographs taken under the microscope. All samples for cell culture were sterilized with UV lamp for 10 minutes.

3. RESULTS AND DISCUSSION

3.1 Formation of the micro-patterned surfaces

The micro-patterned surface was prepared as shown schematically in Figure 2. First, we performed ion-beam irradiation with a stainless mask on sample surfaces at an energy of 150 keV with a fluence of 1×10^{15} ions/cm^2. Second, irradiated layer was formed on the sample surface. Third, we fixed irradiated PLLA to the bottom of a dish filled with PBS (-) solution (pH=7.4) and let it stand at 37 °C in a humidified atmosphere with 5% CO_2. Then, the micro-patterned chip was spontaneously detached from the sample surface and the micro-patterned surface was easily formed (Fig. 3).

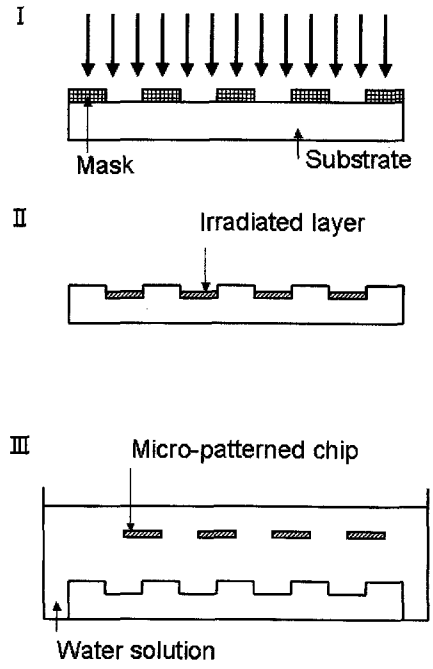

Fig. 2. Schematic diagrams for formation of the micro-patterned surface and micro-patterned chips

Micro-patterning of stainless-steel mask was clearly decaled on the sample surface. Ion-beam irradiation induced depressions on the sample surface in comparison to a non-ion-beam irradiated area. The depth of the depression induced by ion-beam irradiation was 1.6 ± 0.1 μm, as determined STMI measurement. In contrast, depression depth from the micro-patterned chip exfoliated surface was 2.7 ± 0.1 μm from STMI measurement. Therefore, micro-patterned chip thickness was estimated as 1.1 μm, resulting from

subtracting depression depth after micro-patterned chip exfoliation from depression depth immediately after ion-beam irradiation.

Although a figure is not shown here, our previous studies show sheet thickness was 1.2 μm as a result of SEM observation [16]. This value was coincides with the above-mentioned value.

The micro-patterned polymeric surface is expected to be useful in formation of *in vitro* co-culture systems.

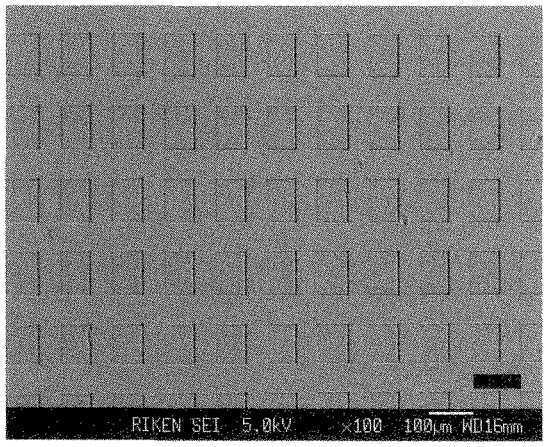

Fig. 3. SEM photograph of a surface micro-patterned by He^+ ion-beam irradiation at 150 keV with a fluence of 1×10^{15} ions/cm^2 after exfoliation in PBS(-) solution. Bar = 100 μ m

3.2 Cell attachment

Figure 4 presents phase-contrast microphotographs of BAECs attachment to He^+ ion-beam irradiated linear domain with a width of 120 μm at 150 keV with a fluence of 1×10^{15} ions/cm^2 after an incubation of 12 hours.

The center of the belt-shaped domain was an irradiated and both outer sides were non-irradiated region. The seeded cells recognized the surface of the ion-beam irradiated region and complete spreading of the cells was observed. It was clearly that BAECs exhibited excellent attachment to the ion-beam irradiated surface.

This result indicated that the surface of ion-beam irradiated PLLA improved cell attachment and appears to be an excellent scaffold in cell culture systems.

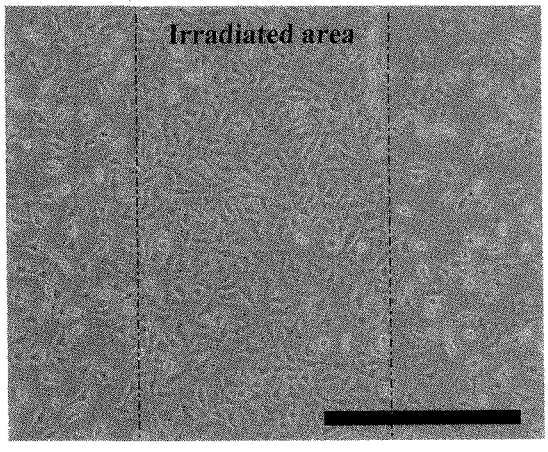

Fig. 4. Phase-contrast photographs of BAECs attachment to a He^+ ion-beam irradiated linear domain with a width of 120 μm at 150 keV with a fluence of 1×10^{15} ions/cm^2 after an incubation of 12 h. Bar = 100 μm.

3.3 Cell growth curve

Figure 5 shows the L929 cell growth curve on the surface of non-irradiated PLLA (square) and on the He^+ ion-beam irradiated PLLA surface (circle) after incubation of three, 17, 24, 48 and 72 hours.

Cell number was plotted as mean±standard deviation (n=3). There was no difference in number after incubation of 3 hours. However there is a recognizable difference in number of cells after 20 hours. It is clearly that the surface of He^+ ion-beam irradiated PLLA promotes cell proliferation.

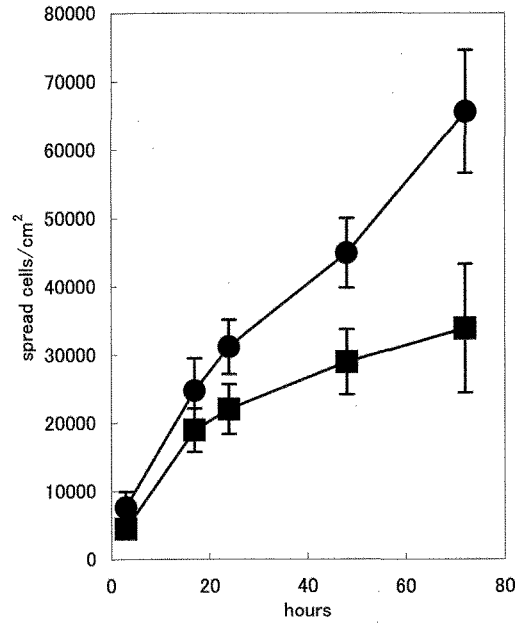

Fig. 5. Cell growth curve on the surface of non-irradiated PLLA (square) and surface of the He^+ ion-beam irradiated PLLA (circle) at 150 keV with a fluence of 1×10^{15} ions/cm^2 after incubation of 3 h, 17 h, 24 h, 48 h and 72 hours. Cell numbers were plotted as mean±standard deviation (n=3).

3.4 Cellular chip and cellular spheroid

Figure 6 shows microphotographs of a micro-patterned cellular chip (a) and cellular spheroid (b).

Micro-patterned cellular chips and spheroids were obtained by the following method. First, we performed ion-beam irradiation with a stainless-steel mask on the sample surface at an energy of 150 keV with a fluence of 1×10^{15} ions/cm^2. A micro-patterned irradiated domain was formed on the sample surface. Second, we fixed irradiated PLLA to the bottom of a dish and seeded BAECs on the sample surface with a culture medium at 37 °C in a humidified atmosphere with 5% CO_2. As a result of this procedure, the micro-patterned cellular chips were spontaneously detached from the sample surface. Any size of self-assembled cellular spheroids with a thickness of 1.1 μm can be obtained by controlling the size of the micro-patterned chip. Spheroid cultivation technology by ion-beam irradiation is useful for cell diagnosis, spheroid cultivation of stem cells, and three-dimensional cell culture.

4. CONCLUSIONS

We investigated the formation of micro-patterned surfaces and micro-patterned cellular chips by ion-beam irradiation into PLLA.

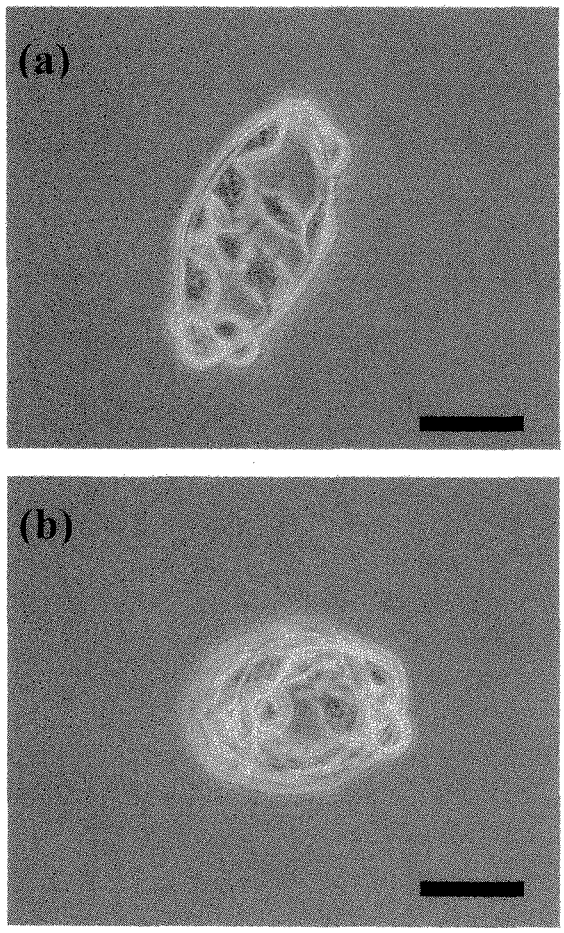

Fig. 6. Phase-contrast microphotographs of cellular chip (a) and cellular spheroid (b). Bar=50 μm

Thickness of the micro-patterned chip was estimated at 1.1 μm, which subtracted the depression depth after micro-patterned chip exfoliation from depression depth immediately after ion-beam irradiation. Surface of ion-beam irradiated PLLA improves cell attachment and promotes cell proliferation.

Any size of self-assembled cellular spheroid with a thickness of 1.1 μm can be obtained by controlling the size of micro-patterned chip.

We previously reported C=C and OH radical induced by ion-beam irradiation were major factors influencing cell attachment [18]. However, one possible explanation for cell attachment to the ion-beam irradiated PLLA is the preferential adhesion of cell adhesive proteins from the surrounding tissue to ion-beam irradiated surfaces. Further studies are required to unravel these effects in more detail.

Ion-beam irradiation onto a PLLA surface is a promising approach for developing new culture systems, such as spheroid cultivation and patterned co-cultures. It is, therefore, very likely that ion-beam irradiation into PLLA will provide new novel methods in regenerative medicine.

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