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Spatial control of cell attachment, proliferation, and differentiation using ion-beam induced thin films

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ABSTRACT

In this study, cellular films were fabricated by ion-beam irradiation into poly-L-lactic acid sheets and cell culture. The cellular film shapes can be controlled by pattern masks. We performed spatial cell patterning using three types of cells: fibroblasts, endothelial cells, and nerve-like cells. First, multi-layered cellular construct was fabricated by stacking fibroblast cellular films. When three cellular films were stacked and incubated, these films firmly attached to each other. Second, tubular constructs were fabricated by endothelial cell culture on linearly patterned surfaces with wide widths of 80, 120, 160, and 200 μm. The patterned cellular films were rounded into vessel-like structure. The diameters of the constructs depend upon the pattern widths. Finally, we controlled cell attachment and nerve growth of nerve-like cells by using linearly patterned surfaces with narrow widths of 10, 30, and 50 μm. Nerve growth direction was controlled by varying the pattern widths. In the case of 10 μm, the attached cells and nerve growth were straight on the patterned thin films. These cell patterning techniques are expected to have applications in tissue engineering, cell transplantation, and in vitro tissue modeling.

Introduction

In natural tissues, many types of cells are highly organized and display tissue-specific functions and structural signals. The spatial organization of cells is critical to generate functional tissues. To construct functional tissues from isolated cells, the cells must be patterned and located on specific regions. Several methods have been used to generate patterns of cells: photolithography, microcontact printing, and microfluidics [1–6]. These patterning techniques are practical for cell patterning on two-dimensional surfaces and have been used for applications in biomaterials science such as tissue engineering, biosensors, and bioassay devices. Cells in tissue and natural organs are three-dimensional environments. What is needed in the context of generating highly organized functional tissues is to transform two-dimensional patterns into three-dimensional structures. Furthermore, it is important that the patterned cells are easy to be handled and transferred to the specific regions.

Ion-beam irradiation has been researched in polymers to regulate cell attachment capability. Cell patterning can be performed by patterned irradiation using a mask, due to improved cell attachment capability [7,8]. Irradiated poly-L-lactic acid (PLLA) sheet exhibits excellent cell attachment capability and a unique phenomenon wherein the irradiated layers exfoliate from the substrate as thin films when immersed in aqueous solution [9,10]. Using this technique, cellular films can be obtained without enzymatic digestion. It was reported that monolayer cell sheet was fabricated utilizing a thermo-responsive polymer, which is easily foldable and wrinkled [11]. In contrast, the cellular films fabricated by ion-beam irradiation are supported by thin films and easy to be handled. The thickness and shapes can be controlled by pattern masks and irradiation conditions [10]. Our previous study also showed that both surfaces of the film were carbonized by ion-beam irradiation, resulting in excellent cell attachment capabilities [12]. The thin films are expected for application to tissue engineering scaffolds and cell transplantation purpose.

In this study, we studied the application of the thin film technique for spatial control of cell attachment, proliferation, and differentiation using three types of cells: fibroblasts, endothelial cells, and nerve-like cells. First, multi-layered cellular construction was formed by stacking fibroblast cellular films. Second,

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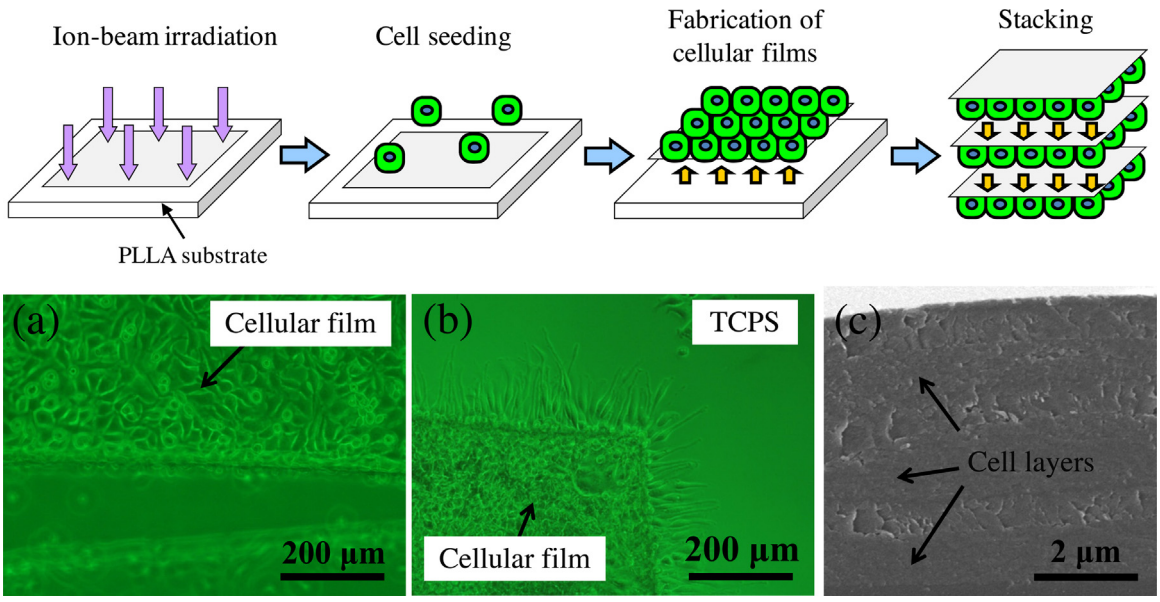

Fig. 1. Schematic diagram of the fabrication of the multi-layered cellular construct. (a) Phase contrast micrograph of L929 attachment on the thin film surface. (b) Phase contrast micrograph of the attachment of L929 cellular film to a TCPS dish. (c) Cross-sectional SEM observation of the cellular construct fabricated by stacking cellular films.

tubular constructions like natural blood vessels were fabricated by wide-patterned irradiation and endothelial cell culture. Finally, we controlled nerve-like cell attachment and nerve growth induction by narrow-patterned irradiation.

Materials and methods

Substrate and ion-beam irradiation

PLLA sheets (Ecoloju; Mitsubishi Jyushi) were cut in a square of 30 mm and used as a substrate. H^+ or He^+ ions were irradiated into PLLA sheets at an accelerated energy of 150 keV with a fluence of 1×10^{15} ions/cm^2. The current density was kept 0.05 μA/cm^2 to prevent heating the substrate. The chamber pressure was less than 5×10^{-4} Pa. Ion-beam was irradiated into a square of 20 mm of the substrate. For patterned irradiation, we used linear-pattern stainless steel masks with width ranging from 10 to 200 μm.

Fabrication of multi-layered cell structure

Mouse fibroblasts (L929; Riken Cell Bank) were cultured in a culture medium (RPMI 1640; Nissui Pharmaceutical) supplemented with 5% fetal bovine serum (FBS; Sanko-Junyaku), and antibiotics. Irradiated PLLA sheets were sterilized under ultraviolet light for 10 min and fixed to the bottom of a tissue-culture polystyrene dish (TCPS; Becton, Dickinson and Company) to prevent floating in the medium. L929 cells were suspended in the medium at a density of 1×10^5 cells/ml, plated onto the irradiated PLLA sheets, and then incubated at an atmosphere with 5% CO_2 at 37 °C. The cells attached and proliferated on the irradiated layer and the irradiated layer exfoliated spontaneously from the original PLLA substrate. The obtained cellular film was transferred to TCPS to evaluate the attachment capability. Cell attachment was observed with an optical microscope equipped with phase contrast objectives, using a CCD camera (IX-70; Olympus). Next, three cellular films were stacked following the procedure: The first layer was placed on irradiated PLLA sheets, and second and third layers were placed on the first layer in turn (Fig. 1). The stacked cellular films were incubated for several hours and cultured again in the medium for 24 h. The cellular construct was fixed with glutaraldehyde (2.5% in PBS) for 2 h, dehydrated by gradient alcohol (30, 50, 70, 90 and 100% ethanol for 10 min each) and then air-dried. After cutting the specimen with microtome, the cross-section was observed by using a scanning electron microscope (JSM-6330F; JEOL).

Fabrication of tubular constructions

Bovine aortic endothelial cells (BAEC) were obtained using dispase cell dissociation. BAEC with passage numbers between 6 and 8 were used in the present experiments. The cells were cultured in the RPMI 1640 medium supplemented with 5% FBS, and antibiotics. After the sterilization and fixation, the cells were suspended in the medium at a density of 1×10^5 cells/ml, plated onto the patterned PLLA sheets with wide widths of 80, 120, 160, 200 μm, and then incubated.

Control of cell attachment and nerve growth induction

Rat pheochromocytoma (PC12) cells are used as a model cell for neuronal differentiation and nerve growth in the presence of NGF. In this study, we used PC12HS cells (Rat adrenal; JCRB Cell Bank), which are NGF-highly sensitive clone of PC12. They were cultured in the RPMI 1640 medium supplemented with 10% heat inactivated horse serum (HS; Invitrogen), 5% FBS, and antibiotics. The cells were suspended in the medium at a density of 1×10^4 cells/ml and plated onto the patterned surfaces with widths of 10, 30 and 50 μm. The cells were cultured for 2 days to attach to the surface and then again cultured for another 2 days in the medium containing 50 ng/ml of nerve growth factor (NGF 2.5S; Invitrogen) to differentiate into neuron-like extensions. The cell attachment and nerve growth were observed with an optical microscope.

Results and discussion

Cellular film stacking

L929 cells were cultured on irradiated PLLA substrates. The cells reached confluent on the patterned films and the irradiated layer was exfoliated from the PLLA substrate at 2 days (Fig. 1(a)). Cell culture was continued up to 6 days. When transferred to TCPS, the cellular film attached to the bottom surface within several hours and the cells began to migrate (Fig. 1(b)). After pouring the culture

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