Interconnection of Mesenchymal Stem Cells Using Regularly Arrayed Wrinkle Microstructures Fabricated by Diamond-like Carbon Thin Film Deposition

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Human mesenchymal stem cells were cultured using poly(dimethylsiloxane) substrates with regularly arrayed wrinkle microstructures formed by partially depositing diamond-like carbon thin films. The optimal design pattern for development of more interconnections of cells within a shorter period was found to be square-shaped areas of 50 μm size arranged at the intervals of 50 - 100 μm in a grid-like pattern.

Keywords: Diamond-like Carbon, Wrinkle microstructure, Mesenchymal stem cell, Network

1. Introduction

As one of engineering factors for developments of therapeutic applications in regenerative medicine, cell scaffolds play a crucial role for controlling cellular activation and various behaviors. For fabricating the cell scaffolds the fibroblast proteins such as collagen included in extracellular matrix (ECM) and polyactic acid have been used as the major structural materials. In recent studies, micro-nano structures of the surfaces have become recognized as the effective inductions of not only cellular attachment, proliferation and shape change but also differentiation [1,2]. Various materials with or formed the micro-nano structures have been investigated as scaffold substrates [3], and differentiation induction mediated by physical stimuli, which can be produced by the substrate structures and the material properties, has become one of the important strategies for improvements of conventional culture methods using chemical agents.

Based on this background, our group focuses on diamond-like carbon (DLC) thin films, composed of mainly carbon and hydrogen, and has investigated phenomena relating to the interface between DLC and cultured cells for the purpose of using the films as scaffold materials. DLC thin films, as medical uses, have been ever applied to the surface coatings of biomedical components such as blood contacting implants typified by heart valves and stents, and tissue contacting implants, taking advantage of the biocompatible, excellent tribological and chemically inert properties [3]. Meanwhile, the studies on application of DLC thin films to cell culturing technologies have been poorly reported, and the films have been mainly investigated in terms of the anti-biofouling property [5]. In this situation, we found techniques to simply obtain DLC thin films having the microstructures on the surfaces by using poly(dimethylsiloxane) (PDMS) elastomer as the substrates. Namely, when the DLC thin film was deposited onto a PDMS substrate directionally stretched in advance, a wrinkle (concavo-convex) microstructure in an array of linear shape was formed on its surface [6,7], and by depositing the film on a PDMS substrate without stretching, a disordered wrinkle microstructure was fabricated on the surface [5,8]. Moreover, from culture tests using mouse fibroblast and myoblast cells it was found that the DLC thin films with wrinkle microstructures had the ability to be more adhered the cells and changed the cellular shape [8-10]. The findings implied that the wrinkle microstructures formed by DLC thin film deposition had the ability to lead to further applications for mediating cell behaviors. Therefore, taking advantage of the wrinkle microstructures, presenting a good cell attachment property, we have conducted studies to control cell shape to develop interconnection between the cells for the purpose of creating a fundamental network structure in order to evaluate neuronal networks.

In this study, as the first step, cell scaffolds with regularly arrayed wrinkle microstructures on the surfaces were formed by partially depositing DLC thin films, and the effects of different design patterns of the wrinkle areas on cellular behaviors, how cells adhere, spread and connect, were investigated to estimate the optimal design for the interconnection.

2. Materials and Methods

2.1 Fabrication of DLC thin film deposited PDMS substrates

The DLC thin films were deposited on flat PDMS plates by an inductively-coupled plasma (ICP) chemical vapor deposition (CVD) system [6]. A plasma generated in the ICP source on the top of the vacuum chamber was irradiated on the substrate holder, which was applied a negative bias voltage with a RF generator. The flat PDMS plate was made by mixing liquid prepolymer (SILPOT 184 W/C: Dow Corning Toray Co., Ltd.) at a ratio of 10:1, and cut off at the size of 70 × 70 × 1 mm after solidification. The PDMS plate directly put on the substrate holder was covered with a metal mask (50 × 50 mm), and deposited the DLC thin film at the conditions: the chamber pressure of 1.3 Pa, the plasma generation RF power of 150 W, the substrate bias voltage of -650 V and the deposition time of 10 min. Before the deposition an argon plasma was irradiated for 10 min for the surface cleaning. In the metal mask used, as shown in Fig. 1, cir-
cle-, square- and triangle-shaped holes (‘size’, diameter or side, of 50 μm) were regularly arrayed in a grid-like pattern respectively, and ‘interval’, the distance between the shapes, was set as 50, 100, 200 and 400 μm, respectively. The 12 patterns with different designs were created together in a sheet of the metal mask. The surfaces of the fabricated substrates were observed using a confocal laser microscope (CLM) (VK-9700, KEYENCE Co.).

2.2 Cell culture tests

Cell culture tests with human bone marrow-derived mesenchymal stem cells (MSCs) (Lonza, PT-2501), were performed using the PDMS substrates deposited the DLC thin films covered with the metal mask above-mentioned. MSCs are cells, which have the multipotent potential to differentiate to lineages of mesenchymal tissues including bone, cartilage and fat, and are relatively easy to be obtained from donors and cultured. The tests were carried out in accordance with the following procedure. MSCs were cultured with a mesenchymal stem cell growth medium (MSCGM) (Lonza, PT-3001) in a 5% CO₂ incubator, and the cells at passage 6 – 9 were used to prepare the cell suspension. The substrates were immersed into ethyl alcohol (99.5%) for sterilization, phosphate-buffered saline (PBS) for rinsing, and collagen solution (type I, 0.01%) for cell attachment. The substrates were put in petri dishes, and the medium followed by the cell suspension were dispensed in them, being a seeding density of 4.0 × 10³ cells/cm². The cells were cultured up to 3 days.

After 1, 2 and 3 days in culture, staining of the cell nuclei by DAPI (PromoKine) and F-actins by Alexa Fluor 488 Phalloidin (Invitrogen) was performed, and the stained cells were observed by a fluorescence microscope (Meiji Techno Co., Ltd., MT6300). Three separate regions of each substrate were photographed. The staining was conducted by the following procedure. The cells were fixed in 3.7% formaldehyde solution in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS at room temperature for 3 – 5 min, and ongoingly incubated using PBS with 1% BSA (bovine serum albumin) at 37 °C for 20 to 30 min. After that, Phalloidin solution was mounted each on the substrates to be left at room temperature for 20 min, and then DAPI solution was mounted each in the same manner to be incubated at 37 °C for 10 to 20 min.

![Fig. 1](image1.png) The design patterns of the metal mask used for DLC thin film deposition on PDMS substrates.

![Fig. 2](image2.png) CLM surface images of the DLC thin film deposited PDMS substrates obtained using the masks with circle, square and triangle-shaped holes arrayed at the intervals of 50, 100, 200 and 400 μm, respectively. Each top-left box presents the magnification of each image at the interval of 50 μm.
3. Results and Discussion

3.1 Substrate observation

Fig. 2 shows the typical surface images by CLM observation as for the DLC thin film deposited PDMS substrates obtained using the masks with circle, square and triangle-shaped holes arrayed respectively at the intervals of 50, 100, 200 and 400 μm. As can be seen in the figure, the same patterns as the masks were observed, and disordered wrinkle structures appeared inside the areas deposited the DLC thin films. Few areas where DLC thin films were exfoliated from the substrate surfaces were confirmed regardless of the shape and the interval of the holes. Our previous studies reported that the wrinkle was constructed from a concavo-convex shape in cross section, and it was confirmed from Raman spectroscopy analysis that DLC thin film was formed by the difference in elastic modulus between DLC and PDMS, and that can be explained by a model assuming the combination of a stiff coating film and a soft substrate \cite{8,31}.

3.2 Cell culture evaluation

From the overall observation of cell nuclei stained, it is clearly indicated that the number of cells on the DLC thin film deposited areas were relatively larger than those on other areas, that is, the surfaces of PDMS, in any cases of circle, square and triangle shapes. The selective attachment of cells on the deposited areas is estimated to be due to the property of DLC thin film to trap cell-adhesive proteins on the surface more effectively compared to the surface of PDMS \cite{8}.

In Fig. 3, the fluorescence microscope images show the F-actins of cells cultured for 1 day on the PDMS substrates having circle, square and triangle-shaped wrinkle areas with 50 μm size arrayed at the intervals of 50, 100, 200 and 400 μm, respectively. With either shape the areas are comparable to a cell size, and can put only a few cells on the surfaces. Therefore, the cells attached on the wrinkle areas showed a tendency to remain at the same places and extend the pseudopodia toward neighbor wrinkle areas. It is noted from the figure, however, that the degree of extension is likely to be weaker in the case of circle-shaped areas and especially larger intervals. For example, as can be seen in the images of 200 and 400 μm intervals of the circle shape, several green-stained circles, being roughly the size of wrinkle areas, are observed. On the other hand, in the cases of 50 and 100 μm intervals, the cells more spread their pseudopodia even any shapes, and for the square and triangle shapes the pseudopodia showed the tendency to extend toward the outside from their vertices of the deposited area shapes.

To evaluate the F-actin-stained images quantitatively, F-actin interconnections between the attached cells on the deposited areas were counted using 3 images obtained respectively from the PDMS substrates having circle, square and triangle-shaped areas arrayed at the intervals of 50 to 400 μm, and that was carried out in each case of 1 to 3 days in culture. The counted interconnection number was divided by the number of the DLC thin film deposited areas seen in each image, and in Fig. 4 the results are organized as ‘Connections per DLC-area’, which means

|     | circle | square | triangle |
|-----|--------|--------|----------|
| 50  | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| 100 | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| 200 | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| 400 | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |

Fig. 3 Typical fluorescence microscope images of F-actins for cells cultured for 1 day on the PDMS substrates having circle, square and triangle-shaped wrinkle areas with 50 μm size arrayed at the intervals of 50, 100, 200 and 400 μm, respectively.
interconnection of cells cultured using the PDMS substrates with DLC thin film partially deposited areas. The optimal design pattern for development of more interconnections of cells in a shorter time was estimated to be the square-shaped areas arranged at the intervals of 50 – 100 μm in a grid-like pattern. This indicates that a moderate distance between the wrinkle areas, not exceeding 100 μm, existed for generating more cellular interconnections, and the appearance of the restriction is suggested to attribute to diffusion distance of signal molecules, which assume cell–cell signaling. And another thing, considering that the pseudopodia showed the tendency to designate the vertices of shapes as their starting points and extend toward the outside of the shapes, as can be seen in Fig. 3, it is suggested that the vertices of square may contribute to the extension of pseudopodia outside the areas. Meanwhile, in the case of circle shape, despite no vertices, larger ‘Connections per DLC-area’ was obtained compared to triangle shape with vertices, especially at the interval of 100 μm and on the second day. This may arise from large difference, over twofold, in areas of circle and triangle shapes. It was actually observed that the circle shape having a larger area attached more cells on the DLC thin film deposited areas, leading to an increase in the number of pseudopodia. Therefore, it is recognized that the results demonstrated that the vertices included in shapes of DLC thin film deposited areas can become one of the potential parameters to control the promotion of cell pseudopodia, but it is necessary to evaluate the effect of vertices solely using different shapes having same areas.

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