Arrangement of PC12 Cells on a Silicon Chip via Extracellular Matrix (ECM) Layer Patterning by Atmospheric Pressure Plasmas

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It is shown that neuronal model cells PC12 (rat adrenal pheochromocytoma cell line) can be cultured on a silicon (Si) substrate with the use of extracellular matrix (ECM) patterned by atmospheric-pressure plasmas (APPs). Arrangement of neuron cells in a desired pattern on a Si chip is an important step for the development of neuron cell chips. In the experiments, 100–200 μm wide strips of ECM (Poly-L-Lysine) layers arranged in parallel were formed on a 1 cm^2 area of a Si surface by the APP etching technique and PC12 cells were shown to grow on the ECM strips. The APP etching technique for ECM layers provides a simple mean of arranging neuron cells on a relatively large area of a Si surface.

Keywords: atmospheric pressure plasma, extracellular matrix, patterning, plasma etching, PC12 cell, cell adhesion

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Control of cell adhesion to and proliferation on a specific surface connected with electrical circuits is a technology required for the development of next-generation biosensors/biochips that house living cells in them. Such sensors and chips, which may be called cell sensors or cell chips, are expected to serve to various applications in biology and medicine. The extracellular matrix (ECM) is key proteins for cell attachment and proliferation. For the arrangement of living cells on a semiconductor substrate, therefore, the formation of patterned ECM layers on the semiconductor surface is an important first step.

Micro contact printing with the use of polydimethylsiloxane (PDMS) stamps [1–3], ink-jet printing with ink-jet printers [4, 5], and micro-fluid techniques [6, 7] have been widely used to control cell attachment and growth on a substrate by patterning ECM proteins. Recently another technique based on patterned etching of ECM layers by atmospheric-pressure plasma (APP) has been proposed [8], which the authors believe offers one of the most cost effective means for ECM patterning.

The goal of this study is to demonstrate that the APP etching method for ECM layers proposed by Ref. [8] can be also applied to the arrangement of neuron cells on a silicon (Si) chip. Specific cells proliferate only on specific ECMs, so the demonstration of HEK293 cells (Human Embryo Kidney cells) on fibronectins (which are used for the ECM for HEK293 cells) presented in Ref. [8] does not automatically guarantee that the same technique can be applied to the growth of different cells on different ECMs. In this study, we shall examine the APP etching technique for the growth of neuron cells on a Si chip, using PC12 cells (Rat adrenal pheochromocytoma cell line) as neuronal model cells and Poly-L-Lysine (PLL) as their ECM. PC12 cells are widely used as neuronal model cells because they extend the cell protrusions such as axons when exposed to the nerve growth factors. PLL is widely used as a scaffold for PC12 cells [9–11] as well as real neurons [12].

In order to grow PC12 cells on a Si chip, we have formed a ECM layer on the chip in the following manner. The surface of a 1.5×1.5 cm^2 Si substrate was cleaned by organic solvent and Piranha clean (H_2O_2/H_2SO_4). Then a SiO_2 layer of 1 μm in thickness was formed on the substrate by thermal oxidation (900°C, 10 h, O_2+H_2O 95°C bubbling). The Si substrate with the oxide layer was then exposed to oxygen plasmas in a plasma cleaner (Harrick Plasma PDC-32G, oxygen gas flow 0.4 SCFH, pressure > 2000 mTorr, 18 W) for 3 min in order to augment hydrophilicity of the substrate surface as well as its affinity to ECM.

PLL is cationic homopolymer of the essential amino acid L-lysine and the molecular weight of PLL used in this experiment (Sigma P4707, 0.01% solution) was 70,000–

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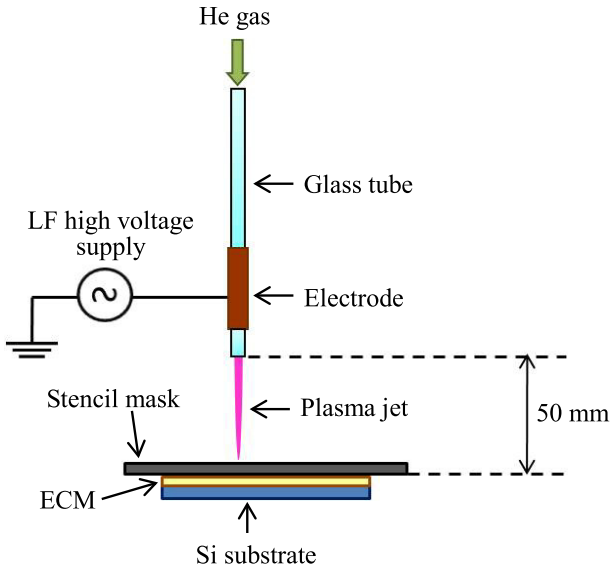

Fig. 1 Experimental set up for ECM patterning by LF APP jets.

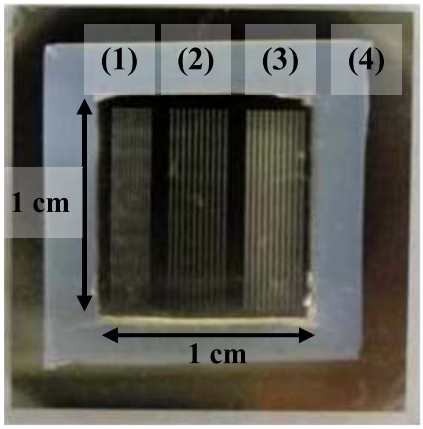

Fig. 2 The stencil mask with slits used in this study.

150,000. More details of PLL may be found in Ref. [13]. For the deposition of PLL, 200 μL of PLL solution was dropped onto the substrate to cover a sufficient area of its surface and let adsorbed overnight in a humidity incubator at a temperature of 37°C and a CO_2 concentration of 5%. Then extra PLL was rinsed off with deionized water and the substrate surface was dried naturally.

For patterning of the ECM layer on a substrate, low-frequency atmospheric pressure plasma jets (LF APP jets) were directly applied to the ECM layer covered by a stainless-steel stencil mask, as shown in Fig. 1. Part of the ECM not covered by the mask is removed by the plasma application and the mask pattern is then transferred to the ECM layer. The technique and the plasma system used in this study are the same as those described in Ref. [8]. Similar plasma systems have also been used for other applications such as sterilization [14].

The LF APP jets are discharges generated in air when a pulsed high voltage is applied to a helium (He) gas flow that flows inside a glass tube. In our experiments, the pulse frequency of power supply was 13 kHz, the inner diameter of the glass tube was 4 mm, and the distance between the sample substrate and the end of glass tube was 50 mm. The tip of the plasma emitted from the glass tube was set to nearly contact the substrate surface during the experiments. LF APP plasma jets were applied to the ECM coated silicon substrate covered with a metal stencil mask.

The metal stencil mask used in this study has three different sets of slits in an area of 1 cm^2, as shown in Fig. 2; (1) ten 100 μm-wide slits with the distance between two adjacent slits of 100 μm, (2) ten 100 μm-wide slits with the distance between two adjacent slits of 200 μm and (3) ten 200 μm-wide slits with the distance between two adjacent slits of 100 μm. The stainless-steel stencil mask was directly placed on the ECM layer of the substrate and pinned down to the substrate by a glue. Optimal conditions for the mask pattern transfer to the ECM layer were obtained by varying the peak-to-peak voltage applied to the electrode, the flow rate of He gas, and plasma irradiation time.

PC12 cells were cultured on the patterned PLL layers formed by the APP etching technique discussed above. PC12 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum and 1% penicillin/streptomycin at 37°C under a 5% CO_2. For cell arrangement, cells were suspended in the fresh medium, and seeded at a density of approximately 7.5 × 10^2 cells/mm^2 on a Si substrate with the patterned ECM layer. The neuronal differentiation by the addition of a nerve growth factor was not applied in this experiment. The cell culture was carried out in accordance with standard cell culture methods (37°C, 5% CO_2). The cellular adhesion, division, and alignment properties on the patterned PLL were observed by a microscope 24 h after the cell seeding.

Figure 3 shows the micrograph of the substrate surface taken 24 h after the initiation of cell culture. The plasma conditions used for the patterning results shown in Fig. 3 are the following: the peak-to-peak voltage was 7 kV, the flow rate of He gas was 4.5 L/min, and the plasma irradiation time was 15 s for uniform scanning of the plasma jets over the mask area of approximately 1 cm^2. Figure 3 (a) shows a surface of the substrate located somewhere in the slits denoted as (1) of the mask shown in Fig. 2, (b) in the slits (2), and (c) in the slits (3), and (d) in the area (4) which was covered by the mask edge and therefore not irradiated by the plasmas. The micrograph Fig. 3 (e) shows a surface of the control substrate (i.e., without plasma irradiation) and Fig. 3 (f) shows a surface of the culture dish. The black dashed lines of Figs. 3 (a)-(c) indicate slit patterns. The positions of slits are indicated by "S" and the positions of the mask (i.e., area between two adjacent slits) are indicated by "M" in Figs. 3 (a)-(c).

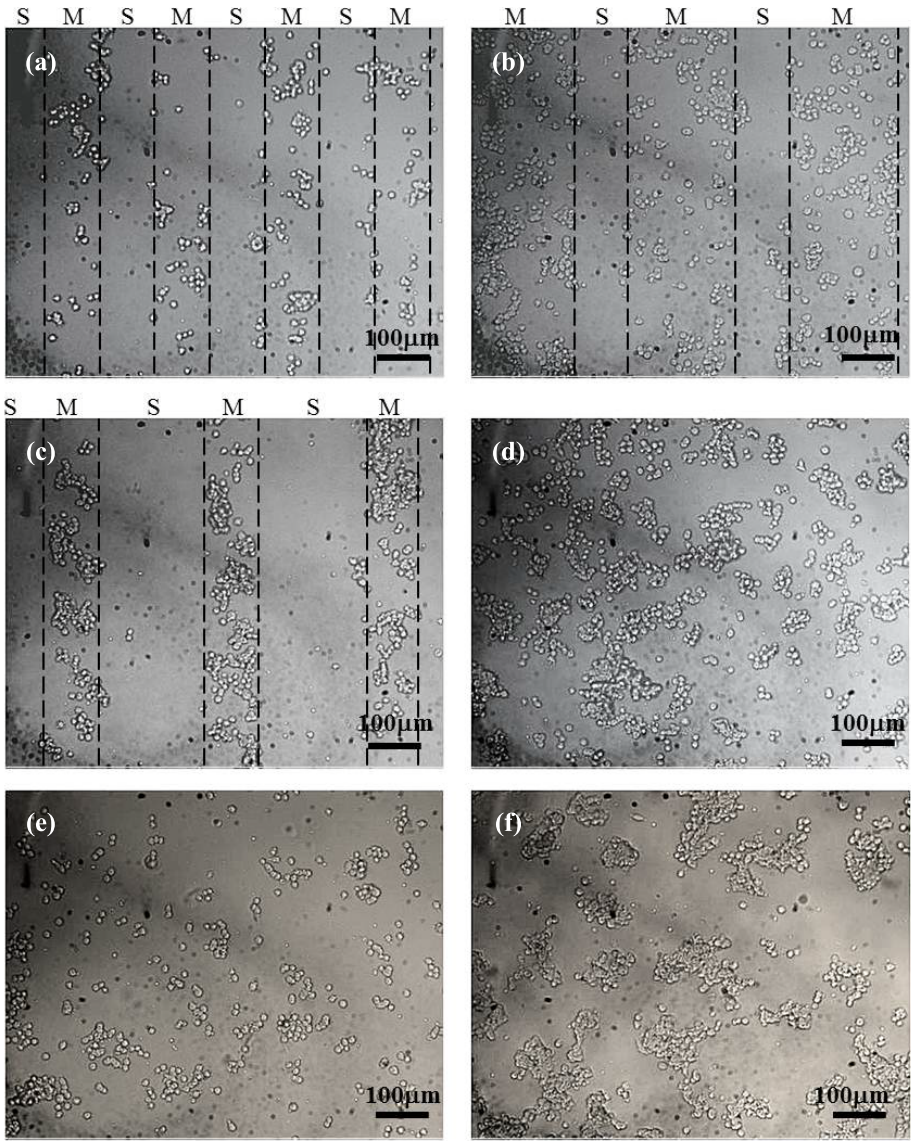

Fig. 3 Micrographs of the cell arrangement patterns on a Si substrate; (a) a surface of the substrate located in the region of slits denoted as (1) of the mask shown in Fig. 2, (b) in the region (2), (c) in the region (3), and (d) in the region of mask edge denoted as (4). The surfaces of the control substrate and of the culture dish are shown in (e) and (f).

It is seen that PC12 cells adhered to and proliferated on the patterned PLL strips in Figs. 3 (a)-(c) whereas the cells are grown at random locations in Figs. 3 (d)-(f). The results indicate that PLL layers covered by the metal stencil mask during the APP etching process do not lose their function as a scaffold for PC12 cells. On the other hand, based on our earlier observations of ECM (fibronectins) surfaces after plasma exposure by Fourier transform infrared spectroscopy (FT-IR) and atomic force microscopy (AFM), which were reported in Ref. [8], we conjecture that the PLL layers were removed after plasma exposure also in the experiments reported here. Although we have not identified the exact cause of desorption of PLL from the substrate surface, it is likely to have reacted with chemically reactive species generated by the plasma and formed volatile species. For example, Deng *et al.* have reported positive correlation between protein destruction and the presence of reactive oxygen species (ROS) generated by helium-oxygen plasma jets [15]. Sakiyama et al. have reported a direct evidence of the presence of ground state oxygen atoms in a helium plasma generated by a plasma needle system with the use of two-photon absorption laser induced fluorescence (LIF) spectroscopy [16]. From these reports, ROS are likely to have played an important role for the removal of PLL also in our system. A close examination to determine the patterning mechanism in our system will be deferred to a future study.

We also note that, in Figs. 3 (a)-(c), a small number of cells ran off the edge of the patterned PLL strips, which indicates the selectivity of PC12 growth on the surface is not perfect under the etching conditions of the present study. If we define the line width roughness (LWR) as the ratio of

a typical distance over which some cells proliferate off the patterned PLL strip to the width of a patterned PLL strip, the LWR under the present experimental conditions can be up to about 40% in the case of 100 μm slits and 100 μm masked strips. Optimization for the LWR will be deferred to a future study.

It has been demonstrated that PC12 cells can be arranged and grown in a pattern of 100–200 μm wide strips separated from each other by similar distances on a Si substrate by means of an APP etching technique for the ECM layer deposited on the substrate. In the etching process, LF APP jets are applied in open air directly to the ECM immobilized on a Si surface through openings of a metal stencil mask mechanically attached to the substrate. Arrangement of PC12 cells in a desired pattern on a Si chip is an important step for the development of cell chips. The APP etching method for the ECM proposed in our study offers a cost effective means for neuron cell proliferation in a desired pattern on a Si chip. Formation of neural networks by the growth of axons of neurons over the patterned ECM is the subject of a future study.

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