Protein patterning by atmospheric-pressure plasmas

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Abstract. Low-frequency (LF) atmospheric-pressure plasma (APP) jets have been used for patterning of thin fibronectin films deposited on a silicon (Si) wafer with the use of a metal stencil mask. Since fibronectin is an adhesion protein that can be found in the extracellular matrix (ECM), a micro-patterned fibronectin film may be used for arranging living cells in a desired pattern on a surface. Removal of fibronectin from the surface by plasma application was observed by Fourier transform infrared spectroscopy (FT-IR) and atomic force microscopy (AFM). It has been found that removal of fibronectin takes place even in the location away from the direct plasma jet application spot, which indicates that desorption of fibronectin is likely to be caused by chemically reactive charge-neutral species that can diffuse away from the plasma without emitting visible light.

1. Introduction

Biosensor and biochip technologies have been widely used for functional analyses of biological materials and systems such as DNAs, proteins and cells. The use of biosensors and biochip is expected to expand further as the technologies are further advanced \([1-3]\). Some biosensors and biochips require micro-patterning technologies to arrange and immobilize biological materials and living cells on a substrate surface while maintaining their biological functions. Especially of interest recently have been micro patterning techniques for large areas as the sizes of biochips continue to increase.

Part of a biochip that serves to transmit and receive electrical signals may be more easily fabricated on a silicon (Si) substrate, to which existing technologies for semiconductor manufacturing can be applied directly for fabrication of such systems. On the other hand, few biological materials or living cells can be immobilized directly on a Si surface. For example, most living cells can be immobilized on a Si substrate only if an extracellular matrix (ECM) layer is placed on the Si substrate. The main functions of ECMS are to provide support and anchorage to cells and to regulate intercellular communication. An ECM is typically composed of proteins such as collagen, laminin, fibronectin, etc.

Micro-patterning of ECMS has been mostly performed so far by micro contact printing (\(\mu\)CP) with the use of polydimethylsiloxane (PDMS) stamps \([4, 5]\) or ink-jet printing \([6]\) with the use of an ink-jet printer. These techniques are widely employed in the biochip research community. With the \(\mu\)CP technique, for example, one can print protein in the grid patterns of several square micrometres on a several square millimetre substrate. However, for some applications, efficient patterning on a larger
substrate (e.g., with the area of several square centimetres) is desirable. As we shall see in this article, protein patterning by atmospheric pressure plasmas (APPs) may provide a solution to such demand.

In the present work, therefore we have examined a possibility of developing a method for ECM patterning on an area of several square centimetres, using low-temperature APP. Such plasmas can be chemically highly reactive and impose little thermal load on the substrate. The method may remind one of the plasma etching technologies widely used in the semiconductor industry. However, the method explored here can be used in air without requiring expensive vacuum equipment. As a mask for patterning, we have used a stainless-steel stencil, a far less expensive alternative to photoresist. Protein patterns and surface characteristics of the substrate after plasma exposure were analyzed by cell culture, Fourier transform infrared spectroscopy (FT-IR) and atomic force microscopy (AFM).

2. Experimental

2.1. Low-frequency atmospheric-pressure plasma jets
Low-frequency (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, as shown in figure 1. The system is essentially the same as those given in Refs. [7, 8]. In our experiments, the peak-to-peak voltage applied to the electrode wound around the glass tube was approximately 7 kV and the pulse frequency was 10 kHz. The flow rate of He gas was 5 L/min. The inner diameter of the glass tube used for protein patterning was 4 mm and that for surface analysis was 8 mm. The distance between the sample substrate and the end of glass tube was set at 20 mm.

![Figure 1. Low frequency atmospheric pressure plasma (LF-APP) jets.](image)

2.2. Sample
In this work, a fibronectin solution (200 μg/ml) was used to form a fibronectin coating of a substrate. Fibronectin, which is found in ECM and body fluids, is a multifunctional glycoprotein composed of subunits with a molecular weight of approx 250 kilodaltons (kDa) [9]. Fibronectin is often used as an ECM when cells are cultivated on SiO₂ substrates [10]. After some quantity of the fibronectin solution was dropped onto the substrate to cover a sufficient area of its surface and let adsorbed for 1.5 hour at room temperature, extra fibronectin was rinsed off by ion-exchanged water. The substrate used in our experiments was either (naturally oxidized) Si for patterning tests and AFM observation or gold for FT-IR analysis.

3. Experimental results

3.1. Protein patterning by LF plasma jets
LF plasma jets were applied to a 15×15 mm square Si substrate coated with fibronectin on which a stainless-steel stencil mask was directly placed. The mask has three different sets of slits, as shown in
figure 2: (1) ten 100 µm-wide slits with the distance between two successive slits of 100 µm, (2) ten 100 µm-wide slits with the distance between two successive slits of 200 µm and (3) ten 200 µm-wide slits with the distance between two successive slits of 100 µm. In the experiments shown in this section, LF jets were applied only to the area (1) (i.e., ten 100 µm-wide 100 µm-apart slits) for 20 sec. After the plasma irradiation, HEK293 cells (Human Embryo Kidney cells) were cultivated on the entire sample surface. Adhesion, division and alignment of cells on the substrate were observed at every 24 hours after cell culture.

Shown in figure 3 is a photograph of the sample substrate surface (without the stencil mask) taken 72 hours after cell culture. Cells are confluenctly cultured on the Si substrate and appear white in colour by the naked eye. Although it is not very clear in figure 3, the area (1) appears darker, which indicates the Si substrate surface is seen and few cells were grown in this area. On the other hand, the white lines observed in the area (3) are cells aligned along the slit patterns. A micrograph of each area is given in figure 4, which shows that few cells indeed exist in the area (1) and cells are only sparsely distributed in the area (2) whereas cells align along the mask slits in the area (3).

Since cells are expected to grow only on the fibronectin coated area, the results shown here indicate that the duration of plasma application was too long, so that fibronectin in the pattern area (1) was completely eliminated even under the mask whereas charge-neutral chemically reactive species (such as gas-phase free radicals) that diffused away from the plasma jet (and therefore were sufficiently diluted in air) patterned the fibronectin film along the mask slits in the area (3).

Figure 2. The stencil mask with slits used in this work.

Figure 3. A substrate surface photograph taken 72 hours after cell culture. LF plasma jets were applied only to the area (1). Cells appear white by the naked eye.

Figure 4. Micrographs of the cell arrangement patterns on the Si substrate of figure 3. The micrograph (a) is in the area (1), (b) in the area (2) and (c) in the area (3). The dashed lines of (c) indicate slit patterns. Cells are expected to grow only on fibronectin-coated areas.
3.2. Surface analysis by Fourier transform infrared spectroscopy (FT-IR)
Fibronectin was deposited on a 13×13 mm square gold substrate which was formed by coating Cr (a few nanometres thick) and Au (45 nm thick) on a glass substrate for FT-IR analysis [11]. LF plasma jets applied to the substrate was scanned uniformly over the entire substrate surface. No stencil mask was placed on the substrate. The plasma application time was varied from 0 sec to 20 sec.

The amid I band spectra (1600 ~ 1700 cm⁻¹) of the fibronectin coating obtained from FT-IR are shown in figure 5 as a function of the plasma application time. The amid I band spectra indicate the presence of the secondary structures such as α-helix, β-turn and β-sheet of protein [11-13]. Also shown by a dotted curve in figure 5 for comparison is the amid I band spectra of the fibronectin coated substrate to which only a He gas flow (without a plasma) was applied for 20 sec. It is shown that, as the plasma-application time increases, the amid I band spectra diminish and nearly vanish at around 20 sec of plasma application. It should be noted that the spectral shape of the amid I band hardly changes as it diminishes, which may indicate disappearance of fibronectin from the surface rather than decomposition of its secondary structures.

3.3. Surface analysis by atomic force microscopy (AFM)
Figure 6 shows the sample substrate surface morphologies observed by AFM (a) before and (b) after plasma application of 120 sec. The entire surface of fibronectin-coated Si substrate was uniformly irradiated by the scanning plasma jets. The average roughness is 3.0 nm for (a) and 0.65 nm for (b). The latter is nearly equal to the roughness of an uncoated Si substrate (which is typically 0.5 nm). From this result together with the FT-IR observation discussed in the previous subsection, it is highly likely that fibronectin is completely desorpted by the plasma application of a sufficient duration time.

4. Discussion
Fibronectin films coated on Si substrates have been patterned by LF plasma jets in this study. Our experimental observations based on cell culture, FT-IR, and AFM have indicated that chemically
reactive gas-phase species (such as free radicals) generated by the plasma are likely to react with fibronectin and remove it from the surface. The reactive species, however, have not been identified in this study.

In the process presented in this work, surface patterns of a fibronectin film are provided by a stainless-steel stencil mask. Since a thin stainless-steel sheet can be easily patterned down to a few dozen micrometres in size by mechanical means, the mask used in our work is inexpensive and reusable. With the use of such a mask, fibronectin directly exposed to the chemically reactive species generated by the plasma is readily removed from the surface whereas fibronectin covered by the mask is less easily removed.

Since the stencil mask is simply placed on the fibronectin film mechanically (with the periphery of the mask pinned down to the substrate by glue), there must be some small spatial gap between fibronectin and the stencil, into which the gas-phase reactive species can diffuse. Therefore, so-called “under-etch”, i.e., removal of fibronectin under the stencil mask was also observed in our study when the plasma exposure time was sufficiently long. The fact that no cell grew in the area (1) of figure 3 is the indication of such “under-etch,” where it seems fibronectin on the substrate surface was completely removed due to overexposure to the plasma despite the presence of a patterned stencil mask.

5. Conclusions
LF plasma jets have been used to pattern fibronectin films deposited on Si substrates. The present study has clearly indicated that a process employing a low-temperature APP with a metal stencil mask is a feasible method to pattern a large area of ECM protein films at least up to several square centimetres. Optimization of plasma application conditions (such as the distance between the plasma jet and the substrate, plasma exposure time, supplied gas specie, and gas flow rate) and mechanism analysis of patterning (influencing radicals and chemical reaction) are deferred to future studies.

Acknowledgments
This work was supported by the Joint Studies Program (2009 ~ 2010) of the Institute for Molecular Science.

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This work was supported by the Joint Studies Program (2009 ~ 2010) of the Institute for Molecular Science.

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