HIGH RESOLUTION PATTERNING OF CELLS WITH A PHOSPHORYLCHOLINE-BASED POLYMER IN A MICROFLUIDIC CHANNEL USING A PARYLENE DRY FILM MASK

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ABSTRACT
This paper describes a method of functionalizing glass surface with a phosphorylcholine (PC)-based polymer that inhibits binding of proteins and cells inside a microfluidic channel (Fig. 1). We coated 2-methacryloyloxyethyl phosphorylcholine (MPC)-polymers on a glass substrate, and then patterned it by an oxygen(O2) plasma using a poly(p-xylene)(Parylene) dry film mask, which is subsequently peeled off from the substrate. We achieved the pattern of cells at the single-cell level, and the array and assay viability of the cells inside a microfluidic channel. This technique of patterning MPC-polymers in the channel will be utilized to characterize cell culture extensively and individually.

KEYWORDS: Phosphorylcholine-based polymer; Dry film mask; Parylene; Cell patterning; Microfluidic channel

INTRODUCTION
Micro-patterning of biomaterials such as cells and proteins is an important technique for drug discovery, biosensor development, and tissue engineering research [1]. Selective surface modification of a substrate using a material that prevents non-specific binding is generally needed to achieve the micro-patterning. For such a purpose, recently, biomimetic phosphorylcholine (PC)-based polymers have attracted attention, because they exhibit an excellent ability to inhibit protein adsorption and cell adhesion due to a large hydration layer [2]. A method using photo-reactive PC polymers and a photo mask has been developed to make micro-patterns of the polymers [3, 4]. However, this technique requires special photo-reactive polymers and the achievable resolution of the pattern is limited.

In this study, we etched a PC-polymer layer by an oxygen (O2) plasma using a Parylene dry film mask that is eventually peeled off from the substrate. We used a commonly available 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer. The advantages of patterning the MPC polymer with a Parylene mask over conventional methods, such as chemical etching, inkjet printing and microcontact printing techniques, include patterning of the MPC polymer in dry condition and uniform and high resolution patterning over a large area. It is because standard lithography was used to pattern micron-sized features in Parylene. In addition, fabrication errors due to air gaps between the substrate and the Parylene mask could be minimized because the Parylene adheres tightly to surfaces.

By taking advantage of the merits of our technique, we produced patterns of the MPC polymer in high resolution inside a polydimethylsiloxane (PDMS) microfluidic channel. In general, an O2 plasma pretreatment of the surfaces is necessary to form a tight seal between a glass substrate and a PDMS microchannel. However, the MPC polymer is ablated in the presence of the O2 plasma. In our method, the Parylene film mask protects the patterned MPC polymers during the plasma treatment. Immediately after the treatment, we peel off the protected Parylene film and superimposed a microfluidic channel over the patterned polymer regions.

Here, we observed the fluorescence of and atomic force microscopy (AFM) images of the patterned MPC polymer after removing the film, and measured cross-sectional profiles of the etched regions of the patterned MPC polymer. In addition, cells were patterned along the micro-patterns of MPC polymer coating. Finally, we performed a viability assay of cell patterns inside a microfluidic channel.

EXPERIMENTAL
Figure 2 shows the process flow of the Parylene mask technique for patterning the MPC polymer inside a microfluidic channel. The first step was patterning an MPC polymer layer on a glass substrate (Fig.2(A)). Then, the patterns inside a microfluidic channel was formed by placing a PDMS channel over the patterned areas and bonding the channel to the glass substrate (Fig.2 (C)). The PDMS channel was fabricated using a photosensitive polymer SU-8 as the mold (Fig.2 (B)). The glass substrate and PDMS channel were exposed to the O2 plasma (25 ml/min, 75 W for 5 sec) before bonding (Fig.2 (A-v),

Combine a pattern of MPC polymers with a microfluidic channel

Peel off Parylene

PDMS channel

Parylene dry film

glass substrate

MPC polymers

cell

Parylene film mask is used to pattern the MPC polymers, and protect it from O2 plasma when combine with PDMS channel permanently for tight sealing

Fig. 1 Conceptional illustration of high resolution cell patterning with biomimetic MPC polymers that prevents non-specific binding of cells using a Parylene dry film in a microfluidic channel.
The substrate and PDMS were brought into contact and heated at 75°C for 1 hour to ensure an irreversible bond (Fig.2 (C)). The Parylene film protected the MPC polymer during exposure to the O₂ plasma and was peeled off in a dry condition just before the bonding. The cells were introduced into the microchannel by inserting or suctioning the cell suspension from a tube with a micro syringe pump (Fig. 2 (D)).

We used bovine carotid artery endothelial cells (ECs) and mouse fibroblast cells (3T3). To visualize the micro-patterns of the MPC polymer, we used rhodamine 6G. The images were captured using a charge-coupled device camera (3CCD Digital Camera C7780, Hamamatsu, Japan) and imaging software (AQUACOSMOS ver.2.6, Hamamatsu, Japan). The topography of the patterned MPC polymer was investigated by AFM (NanoWizard, JPK Instruments, Germany) with a probe (CONT-10, spring constant: 0.2 N/m, resonance frequency: 13 kHz, NanoWorld, Switzerland).

RESULTS AND DISCUSSION

Patterning of the MPC polymer with high resolution

Figure 3 shows fluorescence images of the patterned MPC polymer. In Figure 3(a), the width of the etched regions of the MPC polymer varies between 3 and 100 µm at a regular spacing of 20 µm. In Figure 3(b), the spacing varies between 1.5 and 50 µm in etched regions with an area of 30 × 30 µm². The most finely etched lines and spacing were 3 and 1.5 µm in width, respectively.

Figure 4 (a) shows the AFM images of the patterned MPC polymer. Figure 4(b) shows the cross-sectional profiles of the etched regions. We found that a sharp contrast was produced between the MPC polymer and the etched regions in dry condition, and it remained when the MPC polymer swelled after immersion in water. The thickness of the polymer in the dry condition was 20.8 nm. After immersion in water, the polymer swelled immediately to an average thickness of 45.5 nm (Fig. 4(c)). These results may not be obtained without patterning the MPC polymer; we believe that the sharp contrast allows us to measure the precise thickness of the layer.

Patterning of the cells

Figures 5 shows ECs in linear and circular islands of different sizes on the patterned substrates. Areas with MPC polymer inhibited cells adhesion, proving that the MPC polymer’s cell repellent property was not affected by the Parylene dry film mask process. We found that the cells adhered and elongated longitudinally on the etched regions, particularly when the width of the pattern was narrower than 5 µm. We achieved cell patterns with line widths as fine 3 µm (Fig. 5(a)). As shown in Figure 5(c), the number of cells in an etched region decreases as the size of the pattern becomes smaller. Single-cell patterning was observed for diameters smaller than 20 µm. We also cultured the ECs on substrates with growth regions separated by MPC polymer spacing of various widths (Fig. 5(b)). Although the minimum achievable MPC polymer spacing between growth regions was as low as 1.5 µm (Fig. 2(b)), cells grew between growth regions when the spacing was narrower.

Fig. 2 Method of patterning an MPC polymer using a dry Parylene film mask technique to pattern cells. (A) (i) Graft MPC polymer; (ii) deposit Parylene film onto the MPC polymer; (iii) deposit and pattern the photosist (PR) and aluminum (Al); (iv) etch both the Parylene and MPC polymer with oxygen (O₂) plasma; and (v) peel off the Parylene film to obtain the MPC polymer pattern. (B,C) Permanent bonding of the PDMS channel with the glass substrate is achieved after exposure to O₂ plasma. (D) High resolution patterning of cells inside the channel.

Fig. 3 Fluorescence images of the micro-patterned MPC polymer stained using rhodamine 6G; (a) etched lines with different widths (w): 3, 5, 10, 30, and 100 µm; (b) etched area of 30 × 30 µm² with different spacing (s): 1.5, 2.5, 5, 10, 20, 30 and 50 µm.

Fig. 4 (a) 3D AFM image of the patterned MPC polymer; (b) magnified AFM images of the rectangular area marked by the dashed lines in Fig. 3(a), and cross-sectional profiles of the polymer after immersed in distilled water. (c) The thickness of the polymer in the dry and wet conditions. The error bars represent standard deviation (SD).
than 5 µm. With 10-µm spacing, the cells initially grew between the patterns, but they were eventually confined by the pattern after 3 days of culture because of the cell-repellent nature of the MPC polymer.

Figure 6 shows an image of patterned 3T3 cells inside a microfluidic channel. We successfully produced arrays of cells with a resolution as high as 5 µm wide lines (Fig. 6 (a)). We found that thin patterns of cells (e.g. line widths of 5 µm) detached when the flow rate exceeded 50 ml/min, whereas in the larger patterns of cells (e.g. line widths of 50 µm and the square islands of 20 × 20 µm² in size) remained attached even in high flow rate conditions. One of the advantages of using a micro channel is the ability to assay cells in a small volume. This is particularly important when the reagents are expensive. To demonstrate this idea, we successfully labeled the patterned 3T3 cells in the channel with fluorescent dyes using the LIVE/DEAD® assay kit, which stains living cells green and dead cells red. We confirmed that all of the patterned cells were alive inside the microchannel (Fig. 6 (b)).

CONCLUSION

In this study, we combined MPC grafting with the Parylene dry film mask technique and achieved micrometer-sized patterns of the MPC polymer on a glass substrate. This technique of selective surface modification enables high resolution patterning of the MPC polymer without the use of harsh chemicals. In addition, by combining this technique with a microfluidic channel, we were able to create high resolution patterns of the MPC polymer inside the microfluidic channel. We believe that the high resolution patterning within the microfluidic channel will be useful for developing complex topologies that imitate biological systems. Moreover, using laminar flow in the microfluidic channel, it is possible to apply different dyes or drugs to different regions of a single cell. This technique will be useful for cell studies and applications to develop novel cell-based assays or drugs.

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REFERENCES


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