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Micropatterning of perfluoroalkyl self-assembled monolayers for arraying proteins and cells on chips

Atsushi Kira [a,b,*], Kazunori Okano [c,d,e,**], Yoichiroh Hosokawa [c], Akira Naito [b], Koh Fuwa [a], Jyunpei Yuyama [a], Hiroshi Masuhara [c,f,***]

[a] Research and Development Division, ULVAC Inc., 2500 Hagizono, Chigasaki, Kanagawa 253-8543, Japan
[b] Graduate School of Engineering, Yokohama National University, 79-1 Tokiwadai, Hodogaya-ku, Yokohama, Kanagawa 240-0085, Japan
[c] Graduate School of Materials Science, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan
[d] Kansei Fukushi Research Center, Tohoku Fukushi University, 6-149-1 Kunimigaoka, Aoba-ku, Sendai, Miyagi 989-3201, Japan
[e] Center for Advanced Science and Innovation, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
[f] Department of Applied Chemistry and Institute of Molecular Science, National Chiao Tung University, 1001 Ta Hsueh Road, Hsinchu 30010, Taiwan

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ABSTRACT

Organosilane self-assembled monolayers (SAMs) with perfluoroalkyl groups (R_f) on glass surfaces were used for arraying proteins and cells on chips. Quartz crystal microbalance measurements confirmed the inhibition of protein adsorption on R_f-SAM-modified surfaces and showed efficient adsorption on hydroxyl-, carboxyl-, and amino group-modified surfaces. The characteristics of R_f-modified surfaces were evaluated using solvent contact angle measurement and Fourier transform infrared (FTIR) spectroscopy. The R_f surface was highly water- and oil-resistant, as inferred from the contact angles of water, oleic acid, and hexadecane. Specific peaks of IR spectra were detected in the region from 1160 to 1360 cm^{-1}. Etching with dry plasma completely exfoliated the R_f-SAM, exposing the underlying intact glass surface. Modification conditions were optimized using contact angle and FTIR measurements. After dry plasma processing, the contact angles of all solvents became undetectable, and the IR peaks disappeared. Micrometer scale protein and cell patterns can be fabricated using the proposed method. Protein adsorption on micropatterned R_f-SAM-modified chips was evaluated using fluorescence analysis; protein adsorption was easily controlled by patterning R_f-SAM. PC12 and HeLa cells grew well on micropatterned R_f-SAM-modified chips. Micropatterning of R_f-SAM by dry plasma treatment with photolithography is useful for the spatial arrangement of proteins and cells.

1. Introduction

Recent interest in "-omics" technologies to comprehensively analyze numerous biomolecules or cells has furthered research on the development of microarrays for microelectromechanical systems for biological applications (BioMEMSs). BioMEMSs involve the use of several types of probes composed of DNA [1–3], proteins/peptides [4,5], or cells [6–11] that are usually arrayed on a chip. The technique of arraying probes on a substrate is one of the key factors necessary for achieving high sensitivity and high reproducibility of microarray analyses. A number of fabrication processes of microarray chips have been developed in order to arrange probes on chip surfaces, including laser processing [6], microstamping [7,8], inkjet printing [9], and photolithography processing [4,10,11].

In order to arrange biological probes on a glass or quartz substrate in a two-dimensional pattern of micrometer scale, it is essential to control the adsorption of the probes. Self-assembled monolayers (SAMs) of organosilane have attracted considerable attention as a base material for patterning the probes. SAMs with long-chain alkyl [4] and perfluoroalkyl [10] residues have been patterned by deep ultraviolet (UV) light exposure through a lithographic mask, which brings about protein adsorption and cell adhesion on predetermined positions on the patterned surface. The UV light exposure results in direct photolysis and secondary decomposition, which is ascribed to the active oxygen species produced by the UV light in air as well as to photobleaching [12].

* Corresponding author at: 2500 Hagizono, Chigasaki, Kanagawa 253-8543, Japan. Tel.: +81 467 89 2062; fax: +81 467 58 1643.
** Corresponding author at: 8916-5 Takayama, Ikoma, Nara 630-0192, Japan. Tel.: +81 743 72 6132; fax: +81 743 72 6139.
*** Corresponding author at: 8916-5 Takayama, Ikoma, Nara 630-0192, Japan. Tel.: +81 743 72 6199; fax: +81 743 72 6139.
E-mail addresses: atsushi_kira@ulvac.com (A. Kira), okano@ms.naist.jp (K. Okano), masuhara@masuhara.jp (H. Masuhara).

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These chemical reactions at the interface between intact and photobleached areas limit spatial resolution.

Fabrication of the chips on which biological samples are analyzed has been extensively studied, but the adsorption of probes on the masked areas of chips remains a serious problem. It is generally accepted that the complete elimination of nonspecific adsorption of probes or cells on chip surfaces is difficult. To produce chips on which cells are arrayed, specific areas need to be constructed on the chip to ensure the adherence of particular cells to the chip surface. Chemicals with the perfluoroalkyl (R_f) group and long alkyl chains are one of the probable materials that can be used to prevent nonspecific adsorption of proteins and cells when fine patterns on the chip are required. The R_f pattern can isolate small areas for individual cells.

In this paper, SAM surfaces were micropatterned by dry plasma processing after photolithographic masking. The basic characteristics of the SAM surface as well as the characteristics of protein adsorption and cell adhesion on the patterned surfaces were evaluated for the purpose of producing protein chips and cell chips. The SAM surface was modified with hydroxyl, carboxyl, amino, or R_f groups, and protein adsorption on the surface was estimated by using a quartz crystal microbalance (QCM). Protein adsorption was completely inhibited only on the R_f-modified surface. The R_f-SAM was applied as a base for micropatterning and was optimized using Fourier transform infrared (FTIR) spectroscopy. Finally, spatially selective protein adsorption and cell cultivation on the pattern were achieved.

2. Experimental

2.1. Materials

Organosilane compounds with R_f residues and molecular weights of 5000–7000 were originally synthesized. 11-Hydroxy-1-undecanethiol, 10-carboxy-1-decanethiol, and 11-amino-1-undecanethiol hydrochloride (Dojin Laboratories, Japan), ovalbumin (OVA) (Cosmo Bio Co. Ltd., Japan), and R-phycoerythrin (Molecular Probes Inc., USA) were used without further purification. PC12 cells (RCB0009) and HeLa cells (RCB0007) were provided by the RIKEN BioResource Center (Japan). Dulbecco's modified Eagle's medium, fetal bovine serum, horse serum, streptomycin, penicillins (GIBCO/Invitrogen, Co., USA), and collagen (Cellmatrix type I-C; Nitta Gelatin Inc., Japan) were used as received without purification.

2.2. Surface characterization by QCM for protein adsorption

A QCM sensing system equipped with a 27 MHz AT-cut quartz resonator (AFFINIX Q4; Initium Inc., Japan) was used. The relationship between the changes in mass (Δm, g) and thouse in frequency (ΔF, Hz), as determined using the AT-cut QCM, follows Sauerbrey's equation (Eq. (1)) [13]:

$$\Delta F = -\frac{2F_0^2}{A\sqrt{\rho_q \mu_q}}\Delta m, \quad (1)$$

where F_0 is the original frequency of the QCM (27×10^6 Hz), A is the area of the gold electrode (4.9×10^{-2} cm^2), ρ_q is the density of quartz (2.65 g cm^{-3}), and μ_q is the shear modulus of quartz (2.95×10^{11} dyn cm^{-2}). In the case of the 27 MHz AT-cut QCM, a decrease in frequency by 1 Hz corresponds to a 0.61 ng cm^{-2} increase in the mass of the gold electrode.

The QCM sensor was photochemically cleaned with UV–ozone before modification of the chips with SAM. In order to produce hydroxyl (OH)-, carboxyl (COOH)-, and amino (NH_2)-modified surfaces, the gold electrodes of the QCM sensor were modified with alkanethiols, namely, 11-hydroxy-1-undecanethiol, 10-carboxy-1-decanethiol, and 11-amino-1-undecanethiol, respectively; these compounds formed SAMs on the surface of the gold electrodes. Modification of the QCM sensors with these alkanethiol compounds was carried out by immersing the sensors in ethanol solution of 1 mM alkanethiol for 24 h at room temperature and then washing the sensors with ethanol and deionized water. To modify the SAM surface with R_f-organosilane, the electrodes were coated with a 220 nm thick SiO_2 layer by performing plasma-enhanced chemical vapor deposition (CVD) with SiH_4 and O_2 gases, using the CC-200 system (ULVAC Inc., Japan). The SiO_2 surface sensor was dipped in a hydrofluoroether solution of R_f-organosilane (0.1 wt%). After the SiO_2 surface sensor was dried for 24 h at room temperature, it was washed with hydrofluoroether.

QCM measurements were carried out at 25 °C in 500 μl 10 mM phosphate buffer (pH 7.4) containing 138 mM NaCl and 2.7 mM KCl. OVA was injected at a final concentration of 0.1 mg/ml into the QCM sensors.

2.3. R_f-SAM micropatterning process and surface characterization

Glass substrates were first photochemically cleaned by treatment with UV–ozone. SAMs of R_f-organosilane were then formed on the glass substrates in the manner described above. The R_f-SAM was masked with a positive-type photoresist, which was micropatterned using the lithography process. Oxygen–plasma processing of the photoresist-patterned substrate was performed under an antenna power of 800 W (bias power: 0 W) in 1.0 Pa for 20 s on NE-550 (ULVAC Inc., Japan). When the substrate was exposed to oxygen–plasma, the R_f-SAM in the areas without photoresist was selectively exfoliated. Finally, the positive-type photoresist was removed by washing with acetone.

The surfaces of various SAMs were characterized by contact angle measurement and FTIR spectroscopy. The contact angles for 2 μl each of deionized water, oleic acid, and hexadecane were measured with an automatic contact angle meter (DropMaster 500; Kyowa Interface Science Co. Ltd., Japan). FTIR-attenuated total reflectance (ATR) spectra were measured with an FT/IR-4200 (JASCO Co. Ltd., Japan). As a sample for FTIR-ATR spectroscopy, an R_f-SAM was formed on a Si substrate on which 200 nm gold and 100 nm SiO_2 layers were stacked. Peak assignment for the FTIR-ATR spectra was performed using an FTIR spectral library (KnowItAll; Bio-Rad Laboratories Inc., USA).

2.4. Protein adsorption and cell cultivation on R_f-SAM micropatterned chips

Protein adsorption on R_f-SAM micropatterned chips was tested using R-phycoerythrin. The chip was immersed for 2 h in 50 mM sodium phosphate buffer (pH 7.4) supplemented with 0.15 M NaCl (phosphate-buffered saline (PBS)) containing 40 μg/ml R-phycoerythrin and then washed well with PBS. Adsorption of R-phycoerythrin on the R_f-SAM micropatterned chip was observed using fluorescence microscopy (IX 71; Olympus, Japan) and a charge-coupled device (CCD) camera.

PC12 cells or HeLa cells (1×10^5 cells; 5 ml) were seeded onto the R_f-SAM micropatterned chip, which was placed in a 30 mm ϕ glass-based dish at 37 °C in an atmosphere of 90% moisture and 5% CO_2. The culture medium Dulbecco's modified Eagle's medium was supplemented with 10% fetal bovine serum, 10% horse serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. Prior to cell plating, the micropatterned R_f-SAM-modified chip was coated with collagen (0.3 mg/ml) by immersing the chip in 1 mM HCl solution (pH 3) for 10 min followed by washing with the same HCl solution and PBS. The cells were cultured for 11 days under the above conditions.

3. Results and discussion

3.1. Surface characterization for protein adsorption

Fig. 1 shows the frequency changes, determined using the 27 MHz QCM, upon the addition of 0.1 mg/ml OVA to hydroxyl-, carboxyl-, amino-, and R_f-SAM-modified surfaces. The frequencies of the hydroxyl-, carboxyl-, and amino-SAM surface sensors were decreased to 512.3, 539.3, and 498.3 Hz, respectively, at 8 min after the injection of OVA. In contrast, the frequency of the R_f-SAM surface sensor was not decreased. OVA was used as it is a representative blocking protein for measurements in biochemical analyses, including analysis of biomolecular interactions [14]. This result indicates that the protein adsorption onto R_f-SAM was completely inhibited, while approximately 300 ng/cm^2 OVA was adsorbed onto the three other SAMs (calculated from Eq. (1)). This value indicates that the density of OVA molecules on the hydroxyl-, amino-, and carboxyl-modified surfaces, but not the R_f-modified surface, was 25 nm^2/molecule. Thus, these surfaces were found to be densely coated with OVA because the diameter of the OVA molecule is 4.7 nm, as calculated from the average density of globular proteins, i.e., 1.4 g/cm^3, and an Mr value of 45,000 Da. Therefore, the characteristics of the R_f-modified surface inhibit protein adsorption.

3.2. Surface characteristics of micropatterned R_f-SAM-modified chips

The surface characteristics of R_f-SAM that inhibit protein adsorption are useful to control the attachment of proteins and cells onto the chip surface. We produced micropatterned R_f-SAM-modified chips by using dry plasma processing with photolithographic masking. The R_f-SAM micropatterned chip was characterized using contact angle and FTIR measurements.

The contact angles of deionized water, oleic acid, and hexadecane on the R_f-SAM-modified surface were 118.2°, 77.8°, and 69.0°, respectively. These consistently large contact angles irrespective of the solvent polarity show that the R_f-SAM-modified surface has low free energy, which may be attributed to the low polarizability of CF groups. Consequently, the modified surface may not adsorb proteins. These water- and oil-repelling characteristics effectively suppress protein adsorption on the chip surface because proteins contain both hydrophilic and hydrophobic groups. On the other hand, the contact angles of deionized water, oleic acid, and hexadecane with the surfaces treated with oxygen–plasma for 20 s decreased to less than 5° (below detectable levels). This means that the R_f-SAM was exfoliated from the chip surface by exposure to oxygen–plasma. The surface treated with oxygen–plasma was highly hydrophilic, and this characteristic enables active protein adsorption, which is exactly opposite to the characteristics of the R_f surface.

R_f-SAM exfoliation in response to treatment with oxygen–plasma was evaluated by FTIR measurement. Fig. 2 shows the FTIR-ATR spectra of the R_f-SAM-modified surface before and after exposure to oxygen–plasma: significant peaks were observed at 1363.43, 1317.14, 1253.50, and 1166.72 cm^{-1} (Fig. 2(A)). These peaks can be attributed to R_f-organosilane because it is well known that absorptions due to the chemical bonds in R_f-organosilane, such as C–F$_{(n)}$ and CF$_2$–CF$_2$ bonds, are assigned to the region ranging from 1160 to 1360 cm^{-1}. When the R_f-SAM surface was treated with oxygen–plasma for 5 s (Fig. 2(B)), the intensities of these peaks were almost halved. The contact angle of deionized water with this surface was 57.5°. When the R_f-SAM surface was exposed to oxygen–plasma for 20 s, the FTIR-ATR spectral peaks of R_f-organosilane disappeared (Fig. 2(C)). This result as well as the results of the contact angle measurements indicate that the R_f-organosilane attached to the surface was completely exfoliated by treatment with oxygen–plasma for 20 s and that the intact glass surface was exposed again. On the basis of the above optimization, the R_f-SAM was micropatterned by exposure to oxygen–plasma for 20 s.

3.3. Protein adsorption and cell cultivation on R_f-SAM micropatterned chips

Protein patterning on R_f-SAM-modified chips was evaluated using R-phycoerythrin and fluorescence analysis. Fig. 3 shows the fluorescence images of R-phycoerythrin adsorbed on a micropatterned R_f-SAM-modified chip. Lines (25 μm width) and circles (30 μm diameter) were periodically created into the R_f-SAM (Fig. 3(A) and (B), respectively). Fluorescence from R-phycoerythrin was observed in the areas where the intact glass surface of the substrate was exposed by treatment with oxygen–plasma. We consider that the R-phycoerythrin adsorption onto the R_f-SAM-modified surface was inhibited. The precision of the patterning was estimated from the roughness of the edges of the fluorescent lines, which were less than a few micrometers wide; this result is comparable to or better than that achieved by previous SAM-

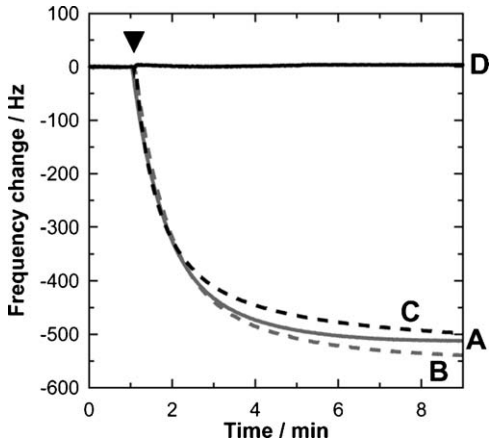

Fig. 1. Frequency changes, determined by a 27 MHz QCM, when 0.1 mg/ml OVA was added to QCM surfaces modified with hydroxyl- (A), carboxyl- (B), amino- (C), or R_f-SAM (D).

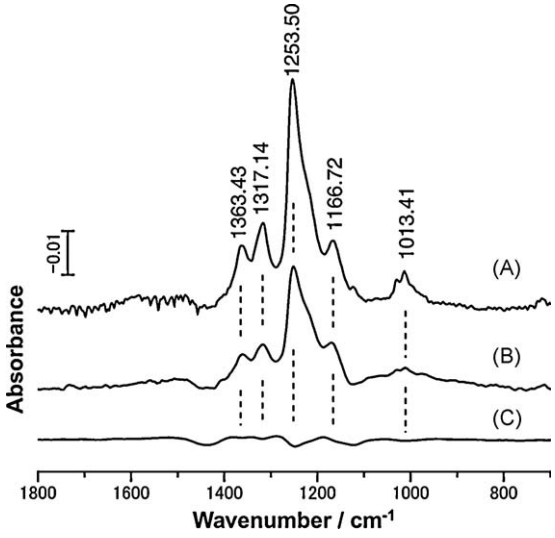

Fig. 2. FTIR-ATR spectra of an R_f-SAM-modified surface (A) and surfaces treated with oxygen–plasma for 5 s (B) and 20 s (C).

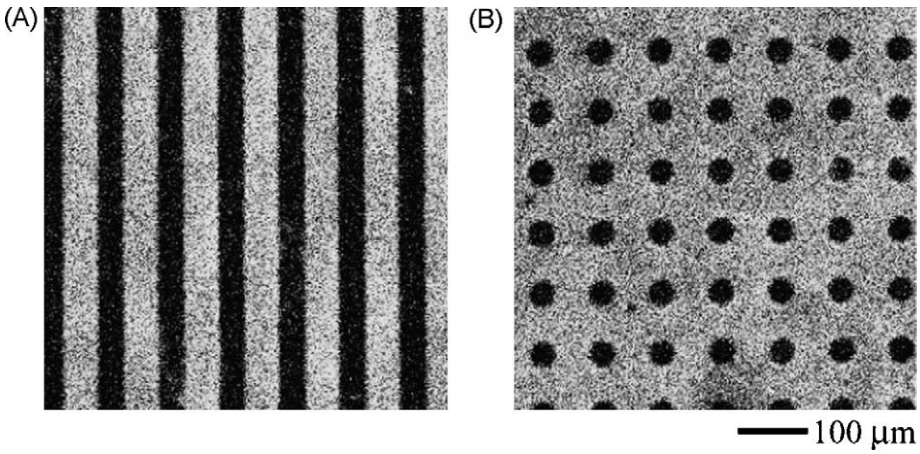

Fig. 3. Fluorescence images of R-phycoerythrin adsorbed on a micropatterned R_f-SAM-modified chip; lines of width 25 μm (A) and circles of diameter 30 μm were periodically created into the R_f-SAM.

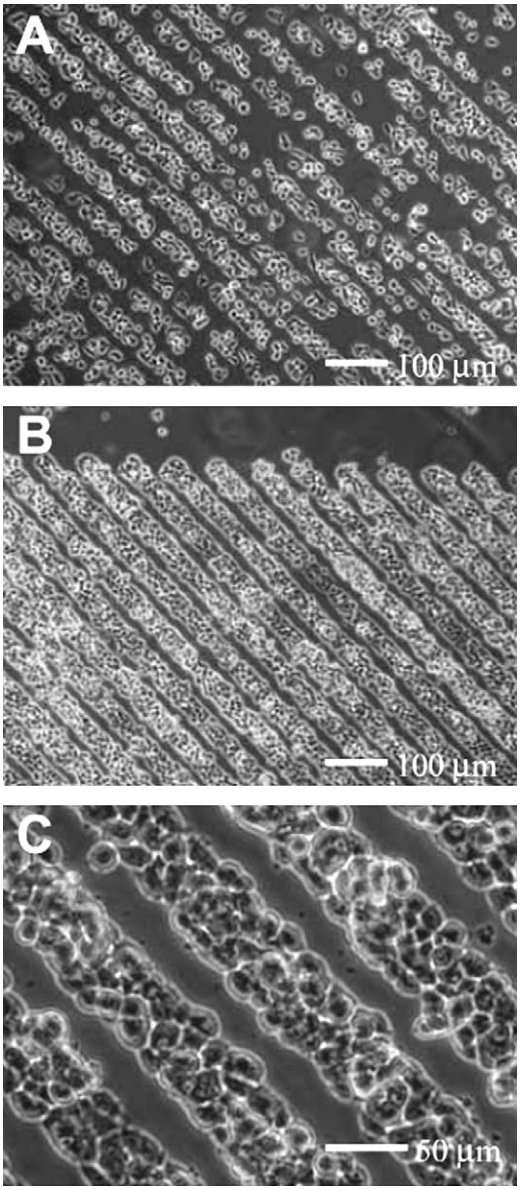

Fig. 4. Microphotograph of PC12 cells on a micropatterned R_f-SAM-modified chip on days 1 (A) and 5 (B and C) after seeding. Lines of width 25 μm were periodically created in the R_f-SAM.

patterning methods that involve exposure to UV light. Degradation of fluorescence intensity was not observed at the edges of the R_f-SAM-modified domains in the micropattern, i.e., the present micropatterning process induced minimal decomposition of the edges.

Finally, cell patterning on the chip with PC12 and HeLa cells was evaluated. Fig. 4 shows lines (25 μm width) of PC12 cells on the R_f-SAM on days 1 (A) and 5 (B and C) after seeding. The cells were selectively adhered onto the areas that were not covered with R_f-SAM. The cell density increased daily because of successive cell division, and the areas without R_f-SAM were completely covered by the cells after 5 days. Since collagen covers only the exposed areas, the cells divided and developed normally in these areas. Furthermore, the patterned cells were well confined within the lines (width, 25 μm) on the R_f-SAM-modified surface. Similar results were obtained with HeLa cells, but some cells formed junctions bridging the Rf-SAM lines, as shown in Fig. 5. The differences in the results are attributable to the characteristics of the applied cells. It is well known that tight junctions exist between HeLa cells. These cells strongly adhere to glass surfaces through extracellular matrix collagen, while PC12 cells only weakly adhere to glass surfaces. PC12 cells are mechanically delicate and are easily removed from the surface to which they are attached. We successfully used both PC12 and HeLa cells to create predetermined patterns on the substrate; therefore, we consider that our micropatterned chip is useful for

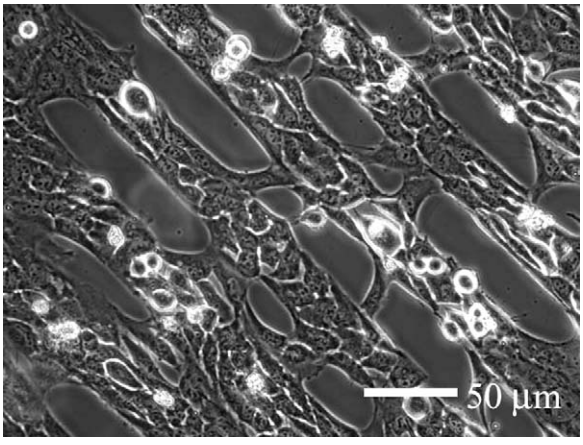

Fig. 5. Microphotograph of HeLa cells on a micropatterned R_f-SAM-modified chip on day 11 of culture.

the study of cell–cell interactions, cellomics, and tissue engineering. The excellent cell patterning ability observed in this study indicates that the collagen adsorbed on the micropatterned R_f-SAM forms a very clear interface at the edges of the line patterns and completely blocks cell emigration.

4. Conclusion

The R_f-SAM was micropatterned by combining dry plasma processing with photolithography. Protein adsorption on R_f-SAM-modified surfaces was completely blocked. The creation of high-contrast micropatterns in R_f-SAMs is indispensable for high-precision arraying of proteins and cells on R_f-SAM-modified chips. The present micropatterning process, which combines dry plasma processing with photolithography, did not induce the decomposition of the R_f-SAM domain edges. Therefore, we demonstrate precise protein and cell patterning, which was comparable to or better than that achieved by previous SAM-patterning methods that involved exposure to UV light [4,10]. This micropatterning method will contribute to the development of high-precision protein and cell chips.

We have also developed an original cell patterning technique using focused femtosecond laser [15,16]. In this method, cells are first cultured on a substrate and then transferred to another substrate by a laser-induced stress wave generated by focusing the femtosecond laser on the culture medium near the source substrate. When the micropatterned R_f-SAM is used as a substrate to transfer cells, several types of cells are individually cultured on the R_f-SAM micropatterns. This type of integrated cell array will soon be reported elsewhere.

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