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Preparation of Self-Organized Micro-Patterned Polymer Films Having Cell Adhesive Ligands

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ABSTRACT: This article describes novel three methods for micro-patterning of cell adhesive ligands by using the self-organized honeycomb-patterned structure formed by the simple cast method. A first method is a direct preparation of a patterned film by casting an amphiphilic polymer containing lactose residue which is one of cell adhesive ligands. A benzene solution of the amphiphilic polymer was cast at high humidity on a glass substrate. Atomic force microscopy (AFM) observation of the film showed that a honeycomb pattern with microporousness with as large as micrometer size in diameter was formed. The film was immersed into an aqueous fluorescence-labeled lectin solution to investigate the distribution of lactoses on the patterned film. Consistence of a fluorescence image of the lectin bound film with the honeycomb pattern showed that the lactose residues were existed not at the holes but at the rims of the honeycomb-patterned film. A second method is to immobilize gelatin, which is one also one of cell adhesive ligands, on the honeycomb-patterned film by chemical reaction. A honeycomb-patterned film was prepared from chloroform solution of an amphiphilic polymer containing reactive succinimide ester groups, and then the film was immersed into an aqueous fluorescence-labeled gelatin solution to introduce gelatin on the film surface. Immobilization of gelatin onto honeycomb-patterned film was confirmed by the fluorescence microscope. A third method is another way to introduce gelatin onto the honeycomb film by the specific avidin-biotin interaction. A honeycomb-patterned film was prepared from amphiphilic polymer containing biotin residues and dodecyl groups, and then the film was immersed into a avidin solution and a biotinylated fluorescence labeled gelatin solution successively. By the fluorescence microscopic observation of the film, gelatin was confirmed to be immobilized at the rims of the honeycomb pattern *via* the avidin-biotin interaction. Cell culture was performed on the gelatin immobilized patterned film prepared by second method. Bioactivity of gelatin immobilized honeycomb-patterned film was confirmed by adhesion of cell onto the film.

KEY WORDS Honeycomb Pattern / Cast Film / Self-Organization / Micro-Patterning / Amphiphilic Polymer / Cell Adhesive Ligand / Avidin-Biotin /

It was reported that cell behaviors such as growth, apoptosis and differentiation are controlled by micropatterned cell adhesive surfaces.[1–4] Hence, micropatterning of cell adhesive regions attracts much attention. A large number of methods for preparing micropatterned surfaces, have been developed, for example photolithography,[5–7] photochemical reaction,[8–12] photodecomposition of self-assembled monolayers,[13–16] microcontact printing[1,17–20] and microfluidic networks.[21–23] In this article, novel method to prepare micropatterns of cell adhesive ligands based on self-organization is described.

We have reported that micrometer size patterned films can be prepared by simple casting of polymer solutions.[24] Regular honeycomb-patterned cast films in micrometer size can be also prepared from amphiphilic polymer solutions under humid atmosphere. This method is significantly simple and dose not require special instrumentation in comparison with methods described above.

Amphiphilic polymers containing functional groups or reactive moieties are expected to form honeycomb-patterned films whose surfaces are covered with cell attaching ligands. For this purpose, copolymers consisting of *N*-dodecylacrylamide[25,26] and functional acrylamide monomer were newly synthesized. Pattern formation of cell attaching ligands was performed by following three methods.

Method 1: the patterned films were prepared directry by casting of the solution of the amphiphilic polymer containing ligand. Copolymer of *N*-dodecylacrylamide and lactose acrylamide (polymer **1**) was newly synthesized. Lactose is known as one of cell adhesive factors.[27–29] The honeycomb-patterned film of the polymer achieved the lactose-patterned structure. Furthermore, molecular recognition of the lactose patterned surface was examined by fluorescence-labeled lectin.

Method 2: the ligand was introduced covalently to the polymer honeycomb pattern. Copolymer of *N*-dodecylacrylamide and the acrylamide active ester was

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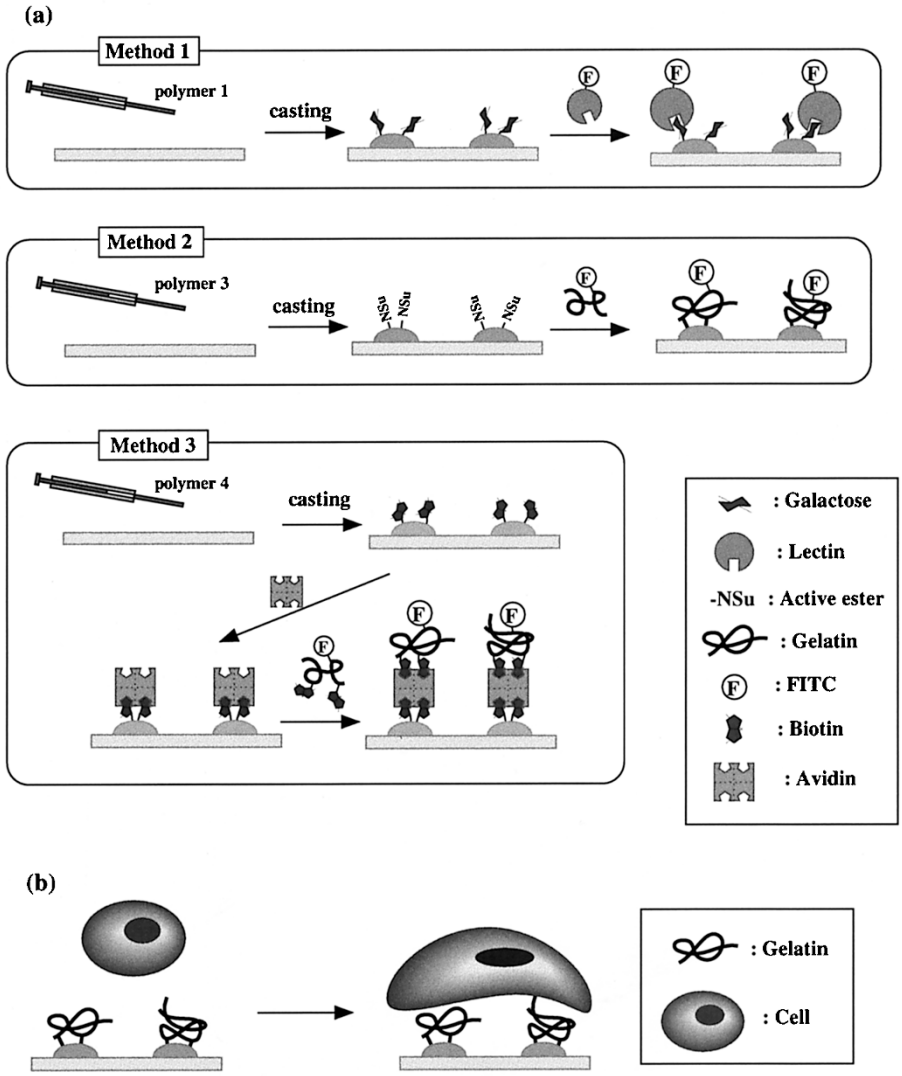


Scheme 1. Schematic representation of (a) procedures for introduction cell adhesive ligands onto honeycomb-patterned films and (b) cell culture on the gelatin immobilized honeycomb-patterned film.

synthesized (polymer **3**). The honeycomb-patterned film containing active ester groups, which enable condensation reaction with amino groups, was prepared from the polymer **3**. By immersion the film into a fluorescence-labeled gelatin solution, gelatin was immobilized onto the honeycomb-patterned film. Immobilization of gelatin on the patterned film was confirmed by fluorescence microscopy.

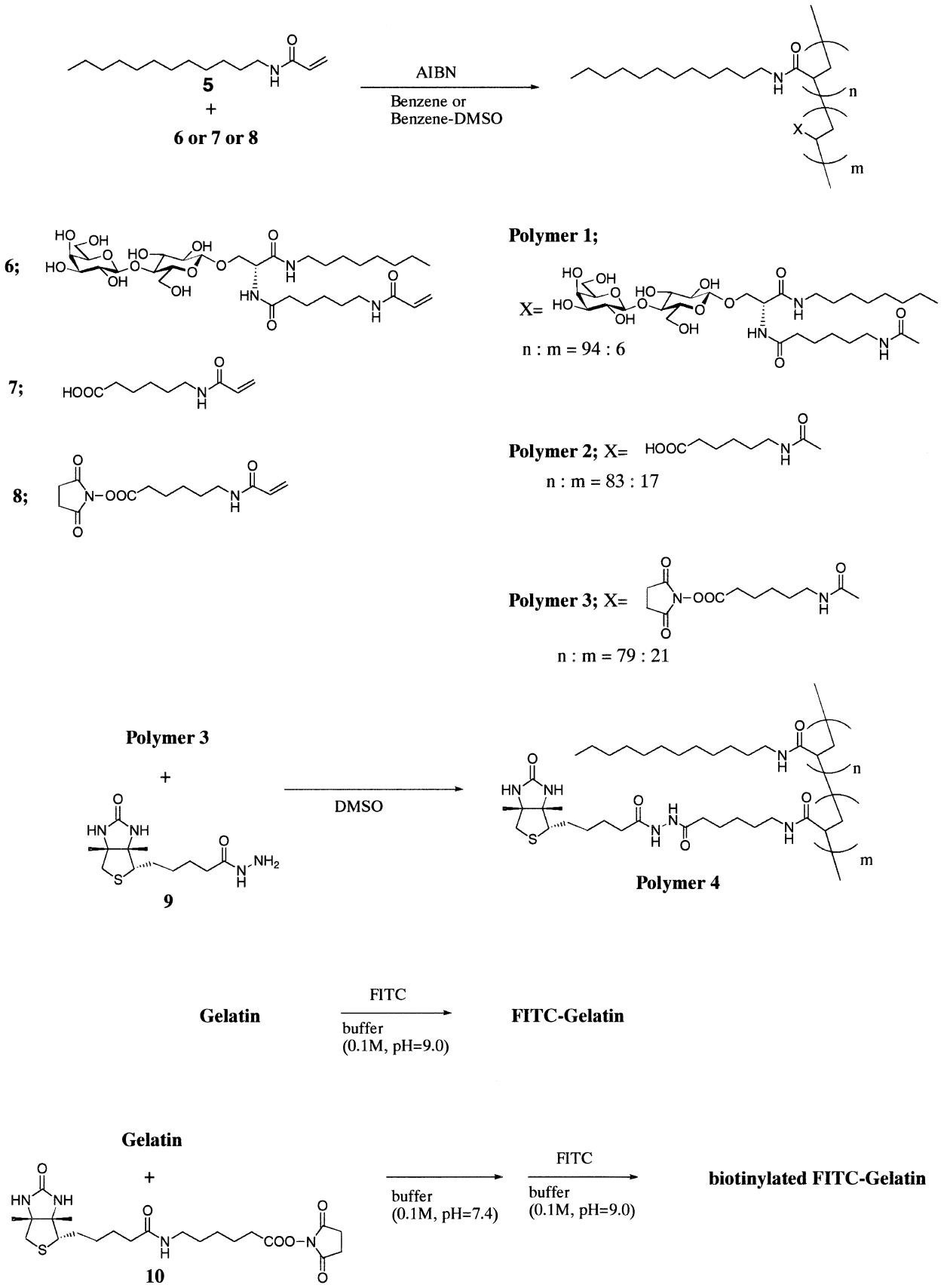
Method 3: the ligand was introduced to the honeycomb pattern *via* the specific interaction of biotin and avidin.[30] A honeycomb-patterned film was prepared from copolymer of *N*-dodecylacrylamide and biotinylated acrylamide (polymer **4**). And gelatin was immobilized by immersion of the film into an avidin solution and then into a fluorescence- and biotin-labeled gelatin solution, sequentially. Patterned immobilization of gelatin was confirmed by fluorescence imaging of the film.

Bioactivity of the honeycomb-patterned films prepared by method 2 was investigated as a function of adhesion of cell onto the films.

EXPERIMENTAL

Materials

Avidin and *Erythrina Cristagalli*-fluorescein isothiocyanate (FITC-ECA) were purchased from EY Laboratories, Inc. Gelatin was purchased from Sigma Chemical Co., Ltd. Glass plates ($18 \times 18 \times 0.15\,mm^3$) were obtained from Matsunami glass Ind., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA), acrylamide, 2,2′-azobis(isobutyronitrile) (AIBN) and solvents were purchased from Wako Pure Chemicals Inc. (Kyoto, Japan). *N*-dodecylacrylamide (**5**), 6-acrylamidehexanoic acid (**7**), *N*-hydroxysuccinimidyl 6-acrylamidohexanoate (**8**), biotin derivatives (**9, 10**), polymerizable lactose derivative (**6**) were synthesized followed by Schnaar,[31] Miyashita *et al.*,[26] Bayer *et al.*,[32] Nishimura *et al.*[33] respectively.


5
+
6 or 7 or 8
AIBN
Benzene or
Benzene-DMSO
n
X
m
6;
7;
8;
Polymer 1;
X=
n : m = 94 : 6
Polymer 2; X=
n : m = 83 : 17
Polymer 3; X=
n : m = 79 : 21
Polymer 3
+
9
DMSO
Polymer 4
Gelatin
FITC
buffer
(0.1M, pH=9.0)
FITC-Gelatin
Gelatin
+
10
buffer
(0.1M, pH=7.4)
FITC
buffer
(0.1M, pH=9.0)
biotinylated FITC-Gelatin

Scheme 2.

Preparation of Amphiphilic Polymers

Radical copolymerizations were carried out under nitrogen atmosphere at 60°C in solutions deaired by three cycles of the freeze-pump and thaw. AIBN was used as an initiator. Molecular weights of the polymers were estimated by the GPC method (Shodex GPC KF-804; Showadenko). The copolymer compositions were determined from the C/N ratio of the elemental analysis.

*Amphiphilic Polymer Containing Lactose Moietie (Polymer **1**).* The lactose monomer **6** (150 mg, 0.21 mmol), *N*-dodecylacrylamide (202 mg, 0.85 mmol) and AIBN (8.7 mg, 53 μmol) were dissolved in benzene-dimethylsulfoxide solution. After stirring the mixture for 24 h, the mixture was poured into large excess amount of acetonitrile. The resulting precipitate was collected by centrifugation (225 mg, 64%). The weight-averaged molecular weight (polystyrene standard) was 1.5×10^4 (M_w/M_n = 2.0). The copolymer was composed of 6 mol% of the lactose monomer and 94 mol% of the dodecyl monomer.

*Amphiphilic Polymer Containing Carboxyl Group (Polymer **2**).* The carboxyl monomer **7** (77 mg, 0.42 mmol), *N*-dodecylacrylamide (400 mg, 1.7 mmol) and AIBN (3.4 mg, 20 μmol) were dissolved in benzene solution. After 24 h, the mixture was poured into large excess of acetonitrile. The resulting precipitate was collected by centrifugation (225 mg, 64%). The weight-averaged molecular weight (polystyrene standard) was 1.2×10^5 (M_w/M_n = 3.9). The copolymer was composed of 17 mol% of the carboxyl monomer, and 83 mol% of the dodecyl monomer.

*Amphiphilic Polymer Containing Active Ester Group (Polymer **3**).* The copolymerization was carried out by the radical polymerization of the active ester monomer **8** (59 mg, 0.21 mmol), *N*-dodecylacrylamide (200 mg, 0.384 mmol), and AIBN (1.7 mg, 10 μmol) in benzene solution. After 24 h, the mixture was poured into large excess of acetonitrile. The resulting precipitate was collected by centrifugation (173 mg, 67%). The weight-averaged molecular weight (polystyrene standard) was 4.2×10^4 (M_w/M_n = 3.3). The copolymer was composed of 21 mol% of the active ester monomer, and 79 mol% of the dodecyl monomer.

*Amphiphilic Polymer Containing Biotynyl Residue (Polymer **4**).* The DMSO solution of polymer **3** (100 mg) and biotin hydrazide (43 mg, 0.17 mmol) was stirred over night at room temperature. The mixture was concentrated and diluted with chloroform. Continuously the solution was washed with 1N sulfuric acid, saturated sodium hydrogen carbonate, and brine then dried over anhydrous sodium sulfate, filtrated, and evaporated *in vacuo*. The residual residue was diluted with chloroform, and poured into acetonitrile. The resulting precipitate was collected by centrifugation (87 mg). The weight-averaged molecular weight (polystyrene standard) was 1.9×10^4 (M_w/M_n = 2.0).

Preparation of FITC-Gelatin

DMF solution of FITC (15 mg) was added dropwise into solution of gelatin (100 mg) in 10 mL of 0.1 M sodium bicarbonate buffer (pH = 9.0). The mixture was stirred at room temperature for 2 h. The reaction was quenched by addition of ammonium chloride (20.6 mg). The resulting solution was dialyzed against water and freeze-dried to give powder (60 mg). Labeling ratio was determined by using adsorption at 494 nm for FITC (ε = 73000 M^{-1}). The ratio of coupled FITC to amino acid residue of gelatin was 1:491.

Preparation of Biotinylated FITC-Gelatin

DMF solution of biotin active ester **10** (18 mg) was added dropwise into solution of gelatin (200 mg) in 40 mL of 10 mM phosphate buffer (pH = 7.4). The mixture was stirred at room temperature for 2 h. The resulting solution was dialyzed against water and lyophilized to give white powder (175 mg). Labeling ratio was determined by use of the 4′-hydroxyazobenzene-2-carboxylic acid (HABA) dye assay.[34] The ratio of coupled biotin to amino acid residue of gelatin was 1:107.

DMF solution of FITC (24 mg) was added dropwise into solution of biotinyl gelatin (80 mg) in 10 mL of 0.1 M sodium bicarbonate buffer (pH = 9.0). The mixture was stirred at room temperature for 2 h. The reaction was quenched by addition of ammonium chloride (33 mg). The resulting solution was dialyzed against water and freeze-dried to give a powder (68 mg). Labeling ratio was determined by using adsorption at 494 nm for FITC (ε = 73000 M^{-1}). The ratio of coupled FITC to amino acid residue of gelatin was 1:46.

Preparation of Cast Film and Introduction of Ligands

Patterned films were prepared by casting of benzene or chloroform solutions of polymers (0.5–1.0 mg mL^{-1}) on glass plates at 25°C and relative humidity of 70–80%. The film prepared from polymer **1** was observed by AFM (NV2100; Olympus).

The film of polymer **1** was immersed into 0.5 mg mL^{-1} solution of FITC-ECA in 0.1 M phosphate buffer solution (pH = 7.3) for 30 min, washed with buffer and water, dried, and observed by a fluorescence microscope (BHS-RFK; Olympus). Fluorescence images were taken by the CCD camera (HCC-3800; Flovel).

The cast film of polymer **3** was immersed into 0.1 mg mL^{-1} solution of FITC-gelatin in 0.1 M phosphate buffer solution (pH = 8.0) for 6 h at room temperature, and then washed with buffer and water, and observed

by fluorescence microscopy.

The cast film of polymer **4** was rinsed with phosphate buffer saline containing 0.02% Tween 20 (PBS/Tween), soaked in BSA solution of PBS/Tween for 30 min for blocking nonspecific absorption. And then the film was incubated in 0.1 mg mL^{-1} solution of avidin in PBS/Tween for 30 min, and 0.1 mg mL^{-1} solution of biotinylated FITC-gelatin in PBS/Tween for 30 min successively. The film was rinsed with PBS/Tween and water, dried and observed by fluorescence microscopy.

Cell Culture

Endothelial cells (ECs), harvested from bovine aorta, were cultured in tissue culture polystyrene dishes. The culture medium was Isocove's modified Dulbecco's medium with 20% fetal calf serum. Cultures were maintained in a 37°C incubator equilibrated with 5% CO_2-95% air. The cells suspended in the Isocove's modified Dulbecco's medium were seeded onto the films of **2** and gelatin-fixed films of **3** prepared on the glass plates. Phase-contrast microscopic images were taken by Olympus IX-70 system after 6 h to observe the adhesion of endothelial cells.

RESULTS AND DISCUSSION

Method 1: Preparation of Honeycomb-Patterned Films of Lactose-Containing Polymer

Preparation of patterned cast films out of ligands containing polymers are regarded as a simple method for micro-patterning of ligands. For this purpose, the amphiphilic polymer **1** containing the lactose residue was synthesized and cast on the substrates in order to obtain a honeycomb-patterned film.

We have reported that the honeycomb-patterned film can be prepared by simple cast of a polyion complex composed of polystyrensulfonate and dimethyldihexadecylammonium under humid atmosphere.[24] The honeycomb-patterned structure is micro-porous with diameter of 2 μm and depth of 300 nm. When a chloroform was used as a solvent, the obtained films of polymer **1** have no regular pattern. However, the honeycomb-patterned film of polymer **1** was obtained by using benzene as a solvent. Figure 1 shows the AFM image of the cast film of polymer **1** at humidity of 80%. AFM observation revealed that the film had the honeycomb structure whose hole diameter and depth was 6 μm and 400 nm, respectively.

The formation process of the honeycomb-patterned film is constituted by following steps. i) Water droplets are condensed at the surface of the casting solution due to cooling by solvent evaporation. ii) Water droplets are closely packed. Fusion of water droplets is inhibited

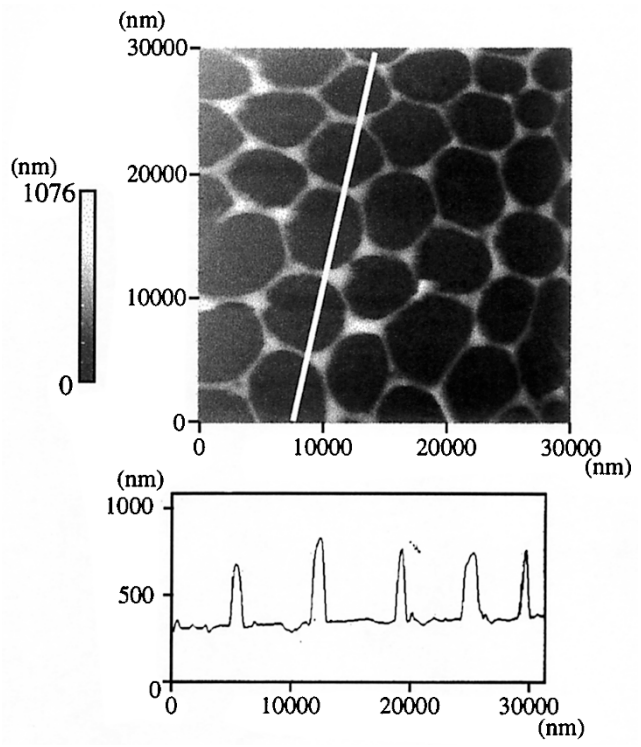


Figure 1. Top view and cross section of AFM image of cast film of **1**.

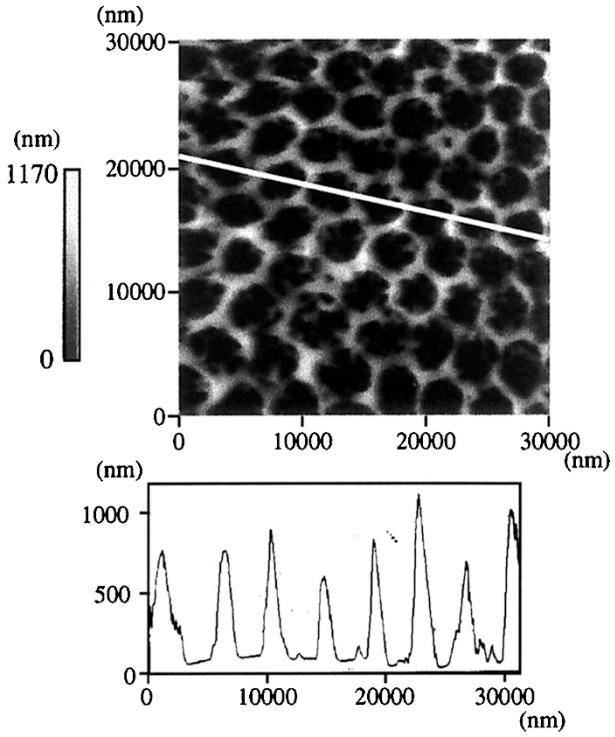


Figure 2. Top view and cross section of AFM image of cast film of **3**.

by polymers adsorbed at a interface between the water droplet and the polymer solution. iii) After solvent evaporation, polymer film having honeycomb structure templated by water droplets is formed. The hole diameter depends on the size of water droplets condensed

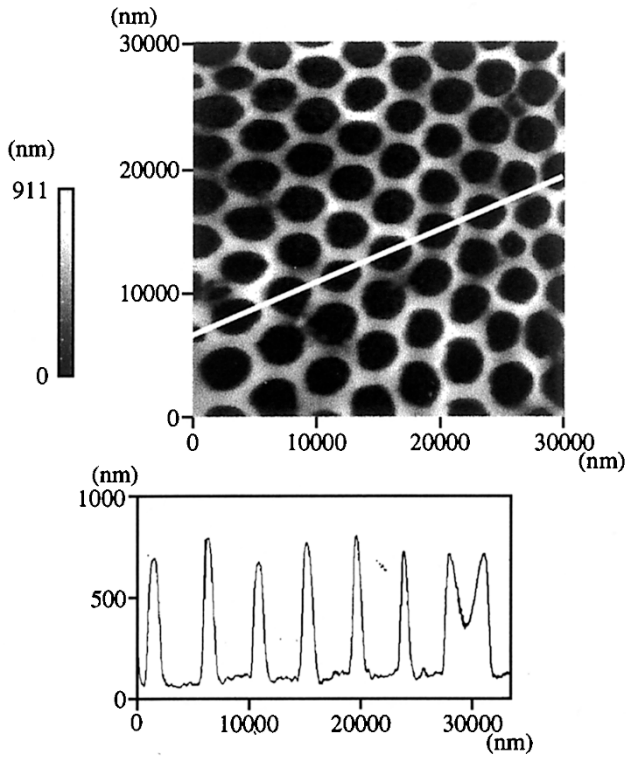


Figure 3. Top view and cross section of AFM image of cast film of **4**.

on the casting solution. Since the water droplets grow up during solvent evaporation, longer evaporation period resulted in bigger diameter of the honeycomb hole than shorter one. Evaporation of benzene takes longer time than chloroform. This must be attributed bigger diameter of the honeycomb hole.

In the forming process of the honeycomb-patterned film, the polymer molecules play an essential role to stabilize water droplets on the solution. Therefore the surface activity of the polymer molecules at the interface between the polymer solution and water droplets is important. The polymer **1** has both of the hydrophobic alkyl chains and the hydrophilic lactose residues. However the polymer **1** is relatively hydrophobic polymer, because a monomer unit ration of the polymer is inclined to the dodecyl acrylamide unit. The surface activity of the polymer **1** is considered to be low. Therefore the water droplets can not be stabilized on the solution surface, and honeycomb-patterned film was hardly formed from chloroform solution.

The reason why the honeycomb-patterned film was successfully formed out of the benzene solution has been unclear yet. Surface energy of the benzene solution of the polymer **1** may be suited for the honeycomb formation.

*Surface Recognition of Honeycomb-Patterned Films of Polymer **1** by Lectin*

Existence of the lactose residue on the patterned film surface was examined by using lectin, which can bind to the lactose residue specifically. The film was immersed into an aqueous fluorescence-labeled lectin (FITC-ECA) solution, washed with a buffer, dried and observed by the transmission and fluorescence microscope (Figure 4). In the transmission image of the polymer film (Figure 4a), the polymer region looks dark due to the different of refractive index between the polymer and the air. The fluorescence image shows that the honeycomb pattern of green fluorescence emission from the fluorescence label was observed (Figure 4b). The polymer region in the transmission image and the fluorescence image of the FITC-ECA were just identical. Apparently, this result indicates that the lactose residues on the patterned film were able to be recognized by the fluorescence-labeled lectin. However, intensity of the fluorescence emission from the bright region was not uniformly. This may suggest that density of the lactose residue is not uniform.

Method 2: Preparation of the Honeycomb-Patterned Film Containing Active Ester Groups and Covalent Bonding of Gelatin onto the Film

Method 1 regarded as a simple and effective way to prepare the honeycomb-patterned films of ligands, because the preparation of the film was achieved by simple casting. However, the synthesis of amphiphilic polymers is difficult owing to the different solubilities between hydrophilic and hydrophobic monomers. Moreover, only low molecular weight ligands can be introduced by chemical modification. So another approach was developed to form ligand patterns. At first, a honeycomb-patterned film containing active ester groups was prepared. Then ligands having amino groups were immobilized on the film surface by the condensation reaction between active ester groups of the honeycomb film and amino groups of ligands.

The honeycomb-patterned film which containing succinimide ester groups was prepared by casting a chloroform solution of the amphiphilic polymer having active ester groups (polymer **3**). Figure 2 shows the AFM image of the film. It was confirmed that the film has the honeycomb-patterned structure. The hole of honeycomb structure has about 3 μm diameter and 600 nm depth. Formation of honeycomb-patterned film is caused by the amphiphilic property of polymer **3** for stabilizing water droplets during the solvent evaporation.

Then introduction of cell adhesive ligands onto the honeycomb-patterned film was performed. For this pur-

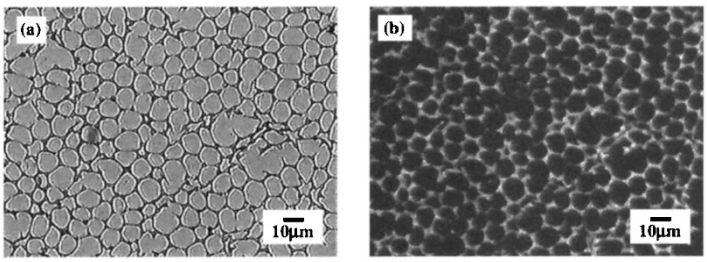


Figure 4. (a) Transmission and (b) fluorescence images of cast film of **1** after binding of FITC-ECA.

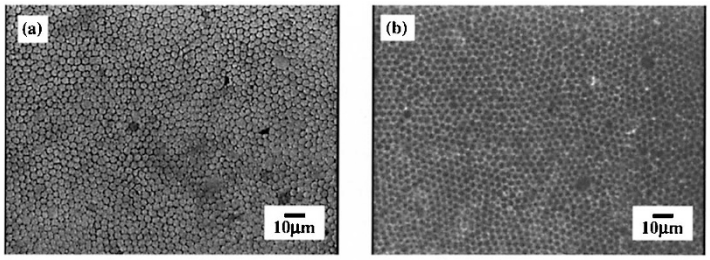


Figure 5. (a) Transmission and (b) fluorescence images of FITC-gelatin fixed cast film of **3**.

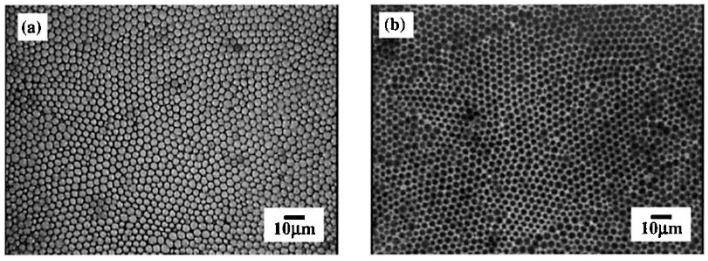


Figure 6. (a) Transmission and (b) fluorescence images of cast film of **4** after sequentially incubated in a solution of avidin and biotinylated FITC-gelatin.

pose gelatin was chosen. Gelatin is one of cell adhesive proteins obtained by hydrolysis of collagen, and widely used for providing materials with cell adhesive property. Since gelatin has lysine residues, arginine residues and terminated amino groups, immobilization of gelatin to the patterned film of polymer **2** would be expected by chemical reaction with the active ester groups. FITC-labeled gelatin was used to confirm the immobilization onto the honeycomb surface by fluorescence microscopy.

The honeycomb-patterned film prepared from polymer **3** was immersed into an aqueous gelatin solution at room temperature for 6 h. The film was washed with a buffer, dried, and observed by the transmission and fluorescence microscopy. A polymer existing region was confirmed by the transmission image (Figure 5a), and a gelatin-immobilized area was confirmed by the fluorescence image (Figure 5b). Comparing these images, selective introduction of gelatin to the rims of the honeycomb structure was indicated. However, ununiformly immobilization of gelatin on the polymer network was also suggested by the ununiformly fluorescence image.

Method 3: Preparation of the Biotin-Containing Honeycomb-Patterned Film and Immobilization of Ligand via Avidin-Biotin Interaction

In order to introduce ligands by an indirect manner, biotin-avidin specific interaction was used instead of the chemical reaction. Avidin is a protein composed of four subunits. Whose each unit have one binding pocket to connect to biotin. And two pairs of the binding pockets face opposite side each other on the avidin molecule. Therefore two kind of biotinylated units can be captured at respective faces at same time. It is expected that a preparation of the biotin-containing honeycomb-patterned film enables to introduce biotinylated ligands onto the honeycomb-pattern surface *via* the biotin-avidin interaction. Since this process carries out under very mild conditions, this method seems to be more utilizable than method 2.

A cast film was prepared from a chloroform solution of polymer **4** containing the biotin residue at high humidity. An AFM image of the polymer **4** film shows that the film has the honeycomb-patterned structure (Figure 3). The hole diameter was about 3 μm and depth was 600 nm. This result indicates that polymer

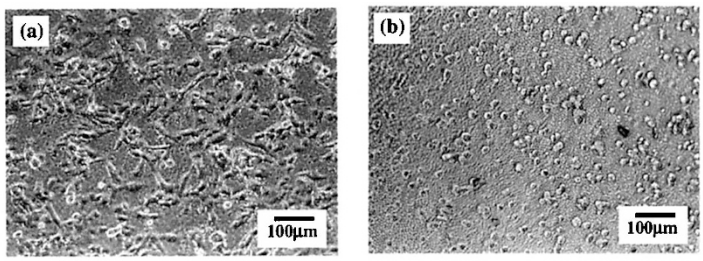


Figure 7. Phase-contrast micrographs of cultured bovine aortic endothelial cells on (a) gelatin immobilized honeycomb-patterned film of **3** and (b) honeycomb-patterned film of **2**.

4 has enough amphiphilic property to stabilize water droplets at the surface of the cast solution and an equal period of time of the chloroform evaporation resulted in equal scale of honeycomb-patterned structure prepared from polymer **3**.

The obtained honeycomb film of polymer **4** was immersed into an avidin solution and then into a biotinylated FITC-gelatin solution, successively. The film was washed, dried, and observed by the transmission and the fluorescence microscopies. Figure 6a shows the transmission image of the film, and the polymer region was confirmed. Figure 6b shows the fluorescence image, and the biotinylated FITC-gelatin immobilized on the biotin-patterned film. Comparing these images, selective introduction of gelatin onto the honeycomb rims *via* biotin-avidin interaction was indicated.

The Bioactivity of the Patterned-Film Having Gelatin

To evaluate the bioactivity of the patterned-film, the cell culture was carried out on the gelatin-immobilized honeycomb-patterned film. In order to measure an effect of the gelatin immobilized on the film, a culture medium without serum was used for seeding cells. Since Serum was absent in media, adsorption of cell attachment factors from media to the film should not be occurred. And the cell attachment was affected by the gelatin immobilized on the surface of patterned films.

When gelatin was immobilized to the active ester surface in buffer solution, hydrolysis of active esters to carboxyl groups might be occurred simultaneously. Therefore a honeycomb-patterned film of polymer **2** which containing carboxyl groups was used as control experiment.

Figure 7a and 7b show phase contrast microscopic images of cells on the honeycomb-patterned films with/without gelatin. It was showed that the cells were extended on the gelatin-immobilized film. On the other hand, cell attachment on the film of polymer **2** was weak and cells retain spherical shapes.

This experiment showed that cell attachment was stronger on the gelatin immobilized honeycomb-patterned film than on the carboxyl groups patterned film. This result indicates that cells recognize immobilized gelatin even on the micro-pattern and cell culture on the patterned film is capable even though the film has rough structure with subcellular scale. The bioactive property can be provided to the honeycomb-patterned films by using this method.

CONCLUSION

This research aims to establish the novel method of micrometer size patterings of bioactive ligands by using self-organized honeycomb-patterned films. For this purpose, three methods have been carried out. Method 1: honeycomb patterning of lactose residues was achieved by the simple cast of a solution of lactose-containing polymer under humid atmosphere. And recognition of lactose residues on the patterned film surface was confirmed as the fluorescence image of the labeld lectin bounded on the surface. Method 2: honeycomb patterning of active ester groups by the cast method and immobilization of gelatin by the condensation reaction were achieved. Method 3: honeycomb patterning of biotin residues by the cast method and pattern immobilization of gelatin by specific biotin-avidin interaction were achieved. Cell adhesion to the gelatin patterned film prepared by method 2 was confirmed by cell culture on the film.

These results show that micro-patterning of ligands can be realized by simple casting method and micro-patterned ligands are recognized by lectin or cell. This micro-fabrication method is considered to be unique, because it depend on self-organization and free from photolithography. This method is significantly simple and dose not require special instrumentation.

It is shown that the gelatin immobilized honeycomb-patterned film has bioactivity and cell culture is capable on the film. In the future, we will culture cells such as hepatocytes which exhibit specific function on the ligand immobilized pattern film.

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