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COLLOIDS AND SURFACES A
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Control of neural stem cell differentiation on honeycomb films

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Abstract

Control of neural stem cell (NSC) differentiation is ongoing interest in neural tissue engineering. Formation of neural networks on various patterned substrates was reported in previous studies. In this study, we cultured NSCs derived from the cerebral cortex of embryonic day-14 mice on honeycomb (HC) films with highly regular pores prepared by casting a polymer solution of water-immiscible solvent under high humidity. The differentiation of NSCs was analyzed by immunostaining for Nestin and MAP2. The differentiation of NSC was controlled for the first time by manipulating the pore size on HC films. The highest suppression of NSC differentiation was observed on HC film with 3 μm pore specifically.

Keywords: Neuron; Neural stem cell; Neural regeneration; Cell differentiation; Micro and nano-pattern

1. Introduction

Controlling cell response to materials is significant for tissue engineering. Topographical patterns of material's surface can affect cell morphology, differentiation, motility and function. Understandably, a variety of biomaterials were investigated for neural tissue engineering application. For example, synthetic materials such as fiber or hydrogels have been used to reconstruct neural network for regenerating neural tissues damaged due to nerve injuries or neurodegenerative diseases [1–3]. Topography of micro-nano patterns on substrates can control direction of neurite outgrowth and neurite development [4–7]. In addition to formation of neural networks, control of NSC differentiation is intriguing topics in neural tissue engineering.

In previous studies, micro-patterns on the surfaces were fabricated by lithography and micro-contact printing [8–13]. These techniques are expected to be applied for neural regeneration in the future. However, the techniques require high energy and involve many processes. In addition, materials for scaffolds applicable to the techniques are limited. We reported that HC films can be prepared by casting a polymer solution of water-immiscible solvent under high humidity on solid substrates [14–16]. This technique has great advantage that the films can be prepared with ease, low-cost and no limitation of materials for scaffold.

This technique was adopted for biological applications. Specifically, the morphology and hence the function of hepatocytes [17,18], cardiac myocytes [19], and endothelial cells [20] were controlled by manipulating the size and shape of the pores on the HC films.

Previous study investigated the effect of a HC film on the formation of neural network [21,22]. We found that neurites extension was controlled by the pores on HC films forming a neural network. In this study, we investigated the possibility to control the differentiation of neural stem cells by manipulating pore size on HC films.

2. Experiment

2.1. Preparation of HC film and flat film

Self-organized HC films were prepared by the method of previous study [16–18]. Briefly, poly (ε-caprolactone) (PCL) and amphiphilic polymer (a copolymer of dodecylacrylamide and ω-carboxyhexylacrylamide (Cap)) were mixed together and dissolved in chloroform (weight ratio of PCL:Cap was 10:1) (Fig. 1). Then, the polymer was cast onto the glass

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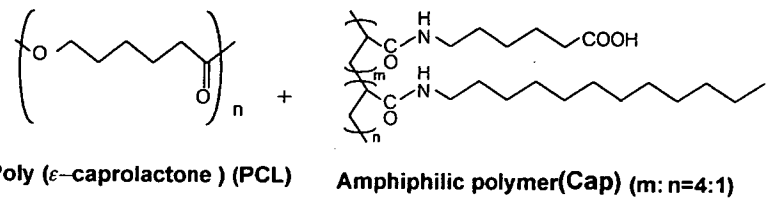

Fig. 1. Chemical structures of poly (ε-caprolactone) (PCL) and amphiphilic polymer (Cap), both of which were used to manufacture the HC-patterned films.

substrates with blowing of highly humid air (1.0 L/min). Flat film was prepared as follows. The polymer solution (poly (ε-caprolactone)/chloroform solution (1 g/L)) was dropped onto a slide glass. The slide glass with polymer layer was span at 1000 rpm for 30 s by spin coater (MIKASA, 1H-7D).

The pore size of the HC film was determined by methods reported previously [21,22]. The flat and HC films were soaked into 1-propanol solution for 5 min and washed with ethanol. Then, the films were attached on micro cover-glass. The films on micro coverglass were put into culture dishes and sterilized by exposing to UV light, followed by soaking into poly-L-lysine solution (50 mg/L, 0.1 M Boric acid, pH 8.3) for an hour to coat poly-L-lysine on the films.

2.2. Cell culture

NSCs were prepared from the cerebral cortex of embryonic day-14 mice (CLEA Japan, Inc). In brief, the cerebral cortexes of embryonic day-14 mice were dissected and the meninges were carefully removed. The tissues were transferred to 15-ml tubes with culture medium containing 55 μM 2-mercaptoethanol and gently triturated with a fire-polished pasteur pipette until most of the tissues were dissociated into single cells. Then, the cell number and viability were measured. The cells were seeded onto the flat film to estimate the population of neural stem cells (NSCs). The neural cells were seeded onto the flat and honeycomb-films at a density of 2.0×10^4 cells/cm^2. They were cultured in serum medium (Opti-MEM (Invitrogen), 10% fetal bovine serum, and 55 μM 2-mercaptoethanol (Invitrogen)) for the first day at 37 °C under a humidified atmosphere of 5% CO_2. After the second day, they were further cultured in serum-free medium (Opti-MEM, B27 supplement (Invitrogen) and 55 μM 2-mercaptoethanol). After 5 days culture, morphologies of neurons and neurites extension were observed using scanning electron microscope (SEM) (Hitachi, S-3500), confocal laser scanning microscope (CLSM) (Olympus, Fluoview FV 300) and phase contrast microscope (Olympus, IX 70).

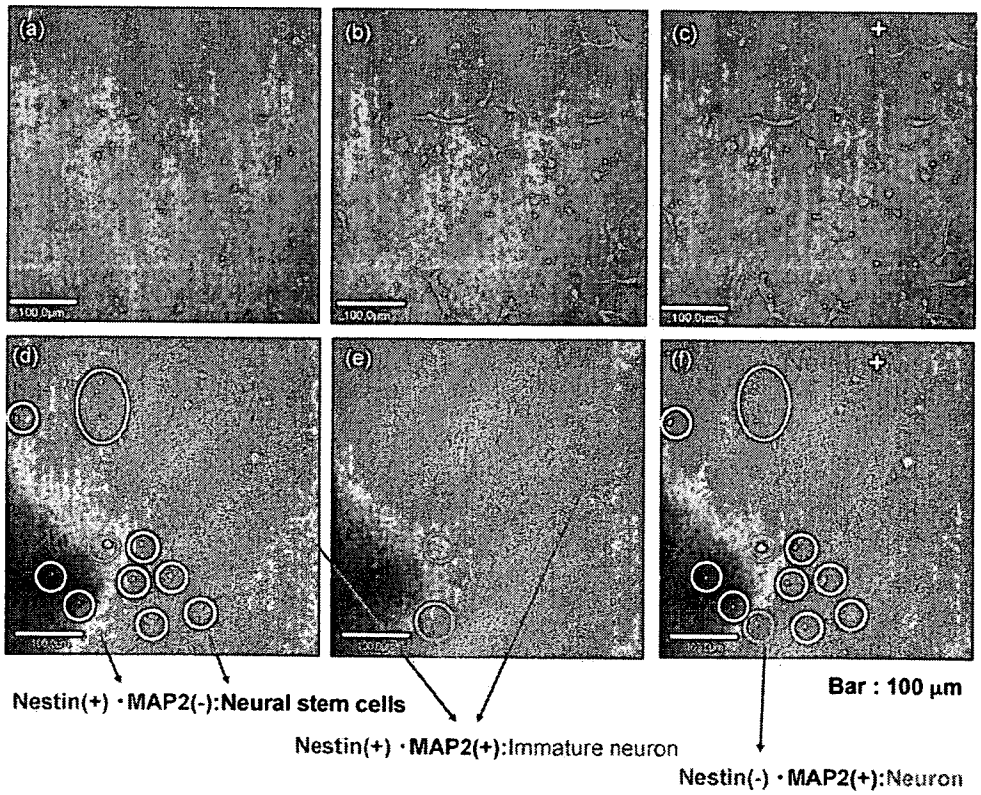

Fig. 2. Immunostaining for Nestin and MAP2 on a flat film and HC films (pore size: 3 μm). (a) Nestin, (b) MAP2, and (c) merge of Nestin and MAP2 on flat film. (d) Nestin, (e) MAP2, and (f) merge of Nestin and MAP2 HC films (pore size: 3 μm), bar: 100 μm.

2.3. Immunostaining for Nestin and MAP2

Cultured cells were fixed with 3.7% formaldehyde in PBS for 30 min at room temperature. Samples were washed with PBS three times for 5 min. Samples were incubated in blocking solution (5% goat serum, 1% BSA, 0.2% Triton X-100 in PBS) for 1 h followed by incubation with mouse monoclonal anti-Nestin (1:1000) and rabbit polyclonal anti-MAP2 (1:1000) in PBS for 2 h. After washing with PBS, cells were incubated with biotinylated anti-mouse IgG (1:1000) for 2 h at 37 °C. Then, cells were incubated with Alexa 488 conjugated avidin (1:1000) and Cy3 conjugated anti-rabbit IgG (1:500) for 2 h at 37 °C. After washing with PBS and water, samples were air-dried and then mounted with mounting media for confocal microscopic observation (OLYMPUS, SV-300).

2.4. Analysis for the degree of cell differentiation

We counted the number of cell positive for Nestin, MAP2 and Nestin + MAP2, respectively on a flat film and HC films (pore size: 3, 5, 8, 10 and 15 μm). Thus, the ratio of positive cell was determined as follows. The ratio of positive cell (%) = (positive cell number/total cell number) × 100. We classified immunostained cells as follows: 1, Nestin(+)/MAP2(−): Neural stem cells. 2, Nestin(+)/MAP2(+): Immature neurons. 3, Nestin(−)/MAP2(+): Mature neurons.

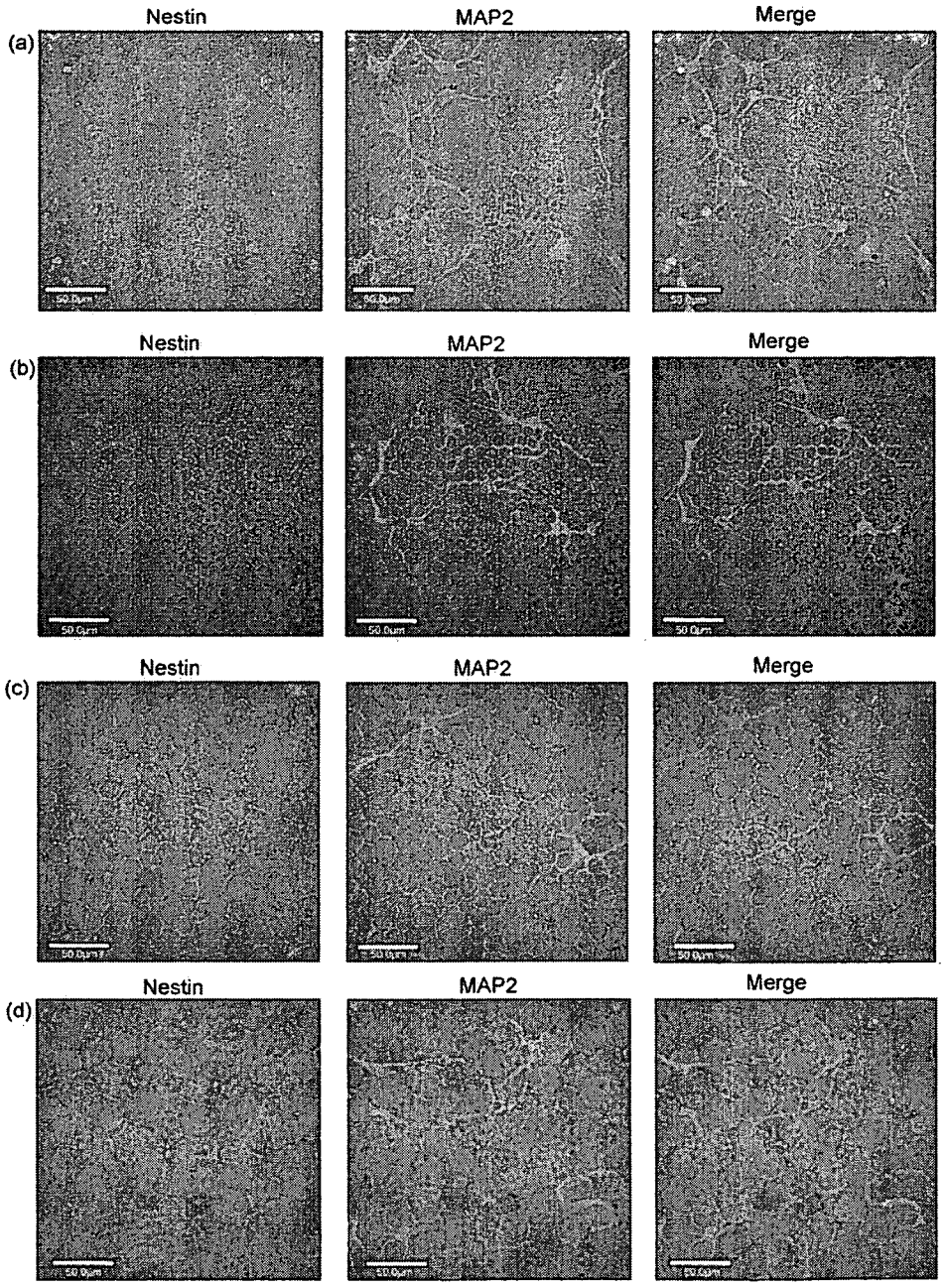

Fig. 3. Immunostaining for Nestin and MAP2 on a flat film and HC films for 4 day culture. Pore sizes (μm) are (a) 5, (b) 8, (c) 10, and (d) 15. Bar: 50 μm.

3. Results and discussion

3.1. Preparation of HC film

We could prepare HC films by casting a polymer solution. The pore size could be controlled in the range from 3 to 15 μm in diameter. The rim of HC films widened with increasing pore size of the films. The porosity of each film was about 50%. In this study, we used the HC films with pore size of 3–15 μm for culturing NSCs.

3.2. Immunostaining for Nestin and MAP2

The cells were characterized by immuno-staining for Nestin, marker of neural stem/progenitor cells and microtuble associated protein 2 (MAP2), marker of neurons after 4 days culture (Figs. 2 and 3).

NSCs cultured for 4 days were negative for Nestin, and positive for MAP2 on a flat film. These results indicated NSCs differentiated into mature neurons for 4 day culture, which extended neurites on a flat film (Fig. 2a–c). On the HC film (pore size: 3 μm), almost all cells were positive for Nestin at day 4. Some cells were positive for Nestin and MAP2 (Fig. 2d–f). Neural stem cells and immature neurons were on the film. These results suggested that NSCs differentiation were suppressed on the honeycomb film. Almost cells were positive for MAP2 and extended neurites on the HC films (pore size: 5, 8, 10 and 15 μm) (Fig. 3a–d). These results showed that almost NSCs differentiated into mature neurons similar to that on the flat film. However, many cells were more positive for Nestin and MAP2 on the HC film (pore size: 5 μm) compared to cells on the HC films (pore size: 8, 10 and 15 μm) (Fig. 3a), showing that almost cell were immature neurons on the HC film (pore size: 5 μm).

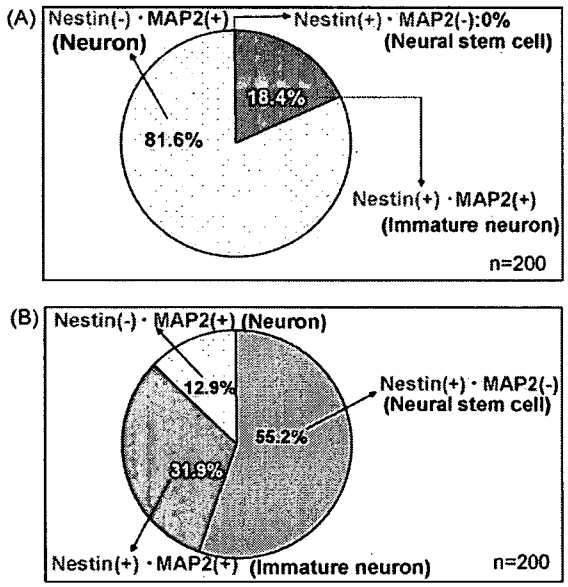

Fig. 4. The distribution analysis of the cells characterized by imunostaining for Nestin and MAP2 on a flat film and HC films (pore size: 3 μm). (A) Flat film, (B) HC film (pore size: 3 μm).

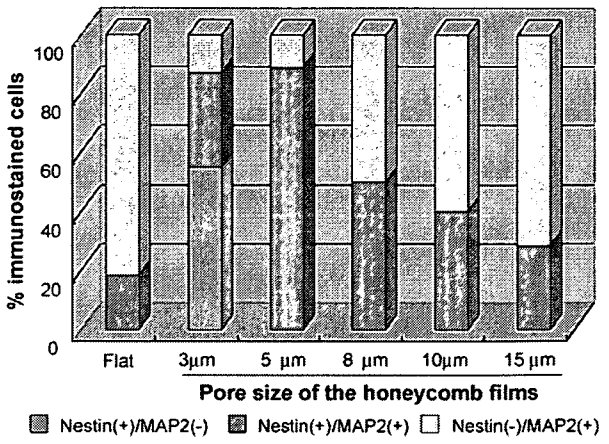

Fig. 5. Dependence of NSCs' differentiation degree determined by immunostaining for Nestin and MAP2 on the pore size of HC films.

3.3. Quantitative analysis of neural stem cell differentiation

We analyzed the degree of differentiation on a flat film and HC films based on the number of cell positive for Nestin and MAP2, respectively. As for the degree of cell differentiation on the flat film, almost mature neurons occupied on the flat film (81.6%) (Fig. 4A). On the other hand, the degree of NSCs, immature neurons and mature neurons were 55.2, 31.9 and 12.9% on the HC films (Fig. 4B). The degree of differentiation indicated NSCs maintain undifferentiated state on the HC film (pore size: 3 μm). The degree of NSC differentiation was depended on the pore size (Fig. 5). The degree of differentiation into neurons was likely to increase gradually as pore size was larger.

We found that NSC differentiation was suppressed by pore size of 3 and 5 μm of HC films. Especially, the HC films (pore size: 3 μm) suppressed NSC differentiation more efficiently than HC films (pore size: >5 μm), and kept NSCs undifferentiated state. On the other hand, almost NSCs differentiated into immature neurons on the HC films (pore size: 8, 10 and 15 μm). These results suggest that topography of HC films influence differentiation of NSCs and development.

NSCs were observed to be trapped on the pore of the HC film (pore size: 3 μm) just after cell seeding. This adhesion arraignment may provide a small adhesion area so that the intercellular signaling of differentiation into neurons may be suppressed in NSCs.

4. Conclusions

This study showed that manipulating pore size on the HC films could control differentiation of neural stem cells. Specifically, HC film with 3 μm pore kept NSCs to be undifferentiated state. This unique feature may be ascribed to the adhesion arraignment of NSCs on HC film with 3 μm pore. The HC films are able to control the differentiation of NSCs and the neurite extension by manipulating the pore size. Thus, HC films are potential materials for neural tissue engineering such as cell therapy and cell transplantation.

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COLLOIDS AND SURFACES A
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Selective metal deposition in hydrophobic porous cavities of self-organized honeycomb-patterned polymer films by all-wet electroless plating

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Abstract

We report selective metal deposition of self-organized honeycomb-patterned polymer (hp) films possessing hydrophobic surfaces by all-wet electroless plating. Nickel/phosphorus (Ni/P) coated hp films were fabricated as follows. A catalytic nucleus of electroless plating was adsorbed on hp films by immersion in a saturated ethanol solution of palladium(II) chloride ($PdCl_2$). Homogeneous metal deposition on the hydrophobic surface of the hp film was easily carried out by all-wet process. In addition an ultrahydrophobic surface of pincushion-structured polymer (ps) films having periodic pillars which was obtained by peeling off a top layer of the hp film was metal-coated by the same all-wet process. It was found that immersion in the saturated ethanol solution of $PdCl_2$ is suitable for catalyzation on hydrophobic surfaces for metal deposition by all-wet electroless plating. To use differences of wettability of honeycomb structures between inner and outer surfaces of porous cavities, site-selective Ni/P deposition in porous cavities was carried out by immersion in a palladium chloride acid aqueous solution after ethanol treatment.

Keywords: Self-organization; Hydrophobicity; Electroless plating; All-wet process; Site-selective deposition

1. Introduction

Ordered metallic structure ranging from submicron to micron scale is one of the significant matters in electronics and photonics. Ordered metallic materials have been fabricated by template synthesis method using organic ordered structures such as lithographed polymer films [1], imprinted polymer films [2], and so on. Recently we have reported that honeycomb-patterned polymer (hp) films were prepared by casting the solution of polystyrene and amphiphilic copolymer **1** (see Fig. 1) by using condensed water droplet arrays as templates [3]. In addition, pincushion-structured polymer (ps) films were fabricated by simple peeling off a top layer of the hp film with adhesive tapes [4].

Ordered metallic layers were prepared by electroless plating of the hp film and the ps film coated by a sputtered platinum/palladium (Pt/Pd) layer [5]. The metallic layer completely reflected the periodic structure of the template films acts as catalytic nuclei carrier of electroless plating. However, the sputtering method gave some disadvantages such as expensive equipment, high running costs, limit of sample size and heating damage of the polymer templates. All-wet metal deposition was used to solve these problems. All-wet metal deposition requires only wet processes including catalyzation, activation, sensitization, metal deposition, and rinse. Commonly used catalyzation process is immersion in a catalytic aqueous solution containing palladium salts. Therefore the template materials must have suitable surfaces for catalyst adsorption such as hydrophilic surfaces [6], chemical-modified surface [7], and physical-modified surfaces [8].

In this report, we demonstrate metallization of the hydrophobic hp and ps films possessing periodic structure by all-wet process (see Fig. 2). All-wet deposition enables us to give wide surface metal deposition easily. In addition, ordered metallic structures were fabricated by site-selective metal deposition of porous cavities of the hp film to use differences of wettability between inner and outer surfaces of the honeycomb structure by all-wet electroless plating.

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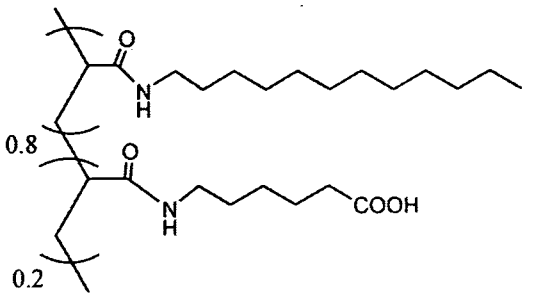

Fig. 1. Chemical structure of amphoteric copolymer **1** used in this study.

2. Experiment

2.1. Preparation of honeycomb-patterned films and pincushion-structured films

The amphiphilic copolymer **1** used in this study was synthesized and characterized according to some previous reports [9]. Honeycomb-patterned polymer (hp) films were prepared by casting a chloroform solution of polystyrene ($M_w = 280\,000\,g\,mol^{-1}$, Aldrich, USA) and the amphiphilic copolymer **1** (10:1 mixture) on a glass substrate with condensed water droplet arrays as templates [9]. Pincushion-structured polymer (ps) films were fabricated by peeling off a top layer of the hp films with adhesive tape [4]. Morphology of the prepared films was observed by a scanning electron microscope (SEM; S-5200, Hitachi, Japan). Contact angles between the polymer films and solutions were measured by contact angle meters (Interfacial Tensiometer PD-W, Kyowa Interface Science, Japan).

2.2. Ni/P coating on the polymer films by all-wet electroless plating

The hp film cut into 15 mm × 30 mm was immersed in 20 ml of a $PdCl_2$ (Kanto Kagaku, Japan) aqueous solution (pH 2.5) adjusted by HCl or a saturated ethanol solution of $PdCl_2$ for 12 h. After rinse with 20 ml of deionized water, the $PdCl_2$-treated hp film was immersed in 20 ml of a nickel/phosphorus (Ni/P) electroless plating bath (pH 5.5) for 24 h at 30 °C. The Ni/P plating bath was containing 0.050 mol dm^{-3} $Ni(H_2PO_2)_2 \cdot 6H_2O$ (Kanto Kagaku, Japan), 0.19 mol dm^{-3} H_3BO_3 (Kanto Kagaku, Japan), 0.030 mol dm^{-3} CH_3COONa (Kanto Kagaku, Japan) and 9.8×10^{-3} mol dm^{-3} $(NH_4)_2SO_4$ (Kanto Kagaku, Japan). The metal-coated films were washed with deionized water and dried naturally. For the ps film, the same treatments were carried out. Surface morphologies of the Ni/P-coated polymer films were observed by SEM.

2.3. Electroless plating in porous cavities of the hp films

The cut hp film was immersed in 20 ml of ethanol to fill the porous cavities, and then immersed in 20 ml of the $PdCl_2$ acidic aqueous solution for 12 h. After rinse with 20 ml of deionized water, the $PdCl_2$-treated hp film was immersed in 20 ml of the Ni/P electroless plating bath for 1 week at 30 °C. Self-supporting ordered metallic structures were obtained by dissolving the template polymer films with chloroform. The surface morphologies of the metallized polymer film and self-supporting metal film were observed by SEM. Chemical compositions of the Ni/P-coated polymer film were investigated by energy dispersive X-ray (EDX; PW-3050, Phillips, Netherlands) analysis.

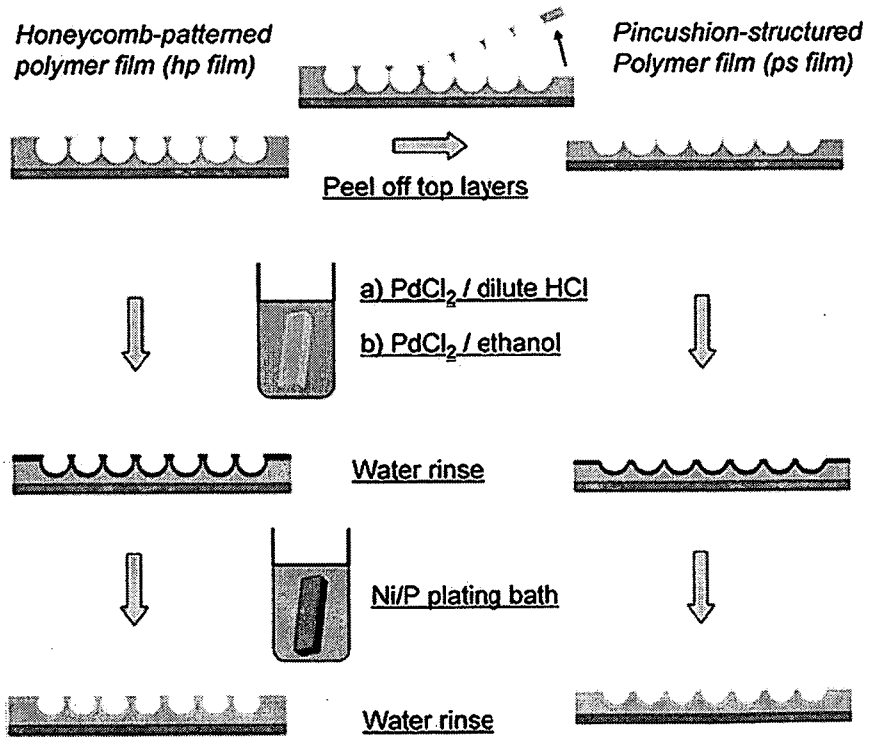

Fig. 2. Schematic illustration of Ni/P coating of the hp films and the ps films by all-wet electroless plating.

3. Results and discussion

3.1. Ni/P coating of the hp films

Fig. 3a indicates an SEM image of the hp film having regular pores of 7.0 μm in a diameter. The pore edge accumulates the amphiphilic compound **1** [10] and layer thickness of the edge is estimated to be less than 0.50 μm by a magnified SEM image of the pore edge as shown in Fig. 3b. Ni/P electroless plating of the hp film was carried out by immersion in a $PdCl_2$ catalytic solution and then in a Ni/P electroless plating bath. Fig. 3c and d show SEM images of Ni/P coated hp films prepared by using the acidic $PdCl_2$ aqueous solution and the saturated ethanol solution of $PdCl_2$ as catalytic solution, respectively. Ni/P metal was partly deposited on the pore edge by immersion in the commonly used acidic $PdCl_2$ aqueous solution. On the other hand, homogenous Ni/P deposition on the hp film was observed when the catalyzation process was carried in the saturated ethanol solution of $PdCl_2$. This difference can be explained by considering wettability between the surface of the hp film and each catalytic solution. In this study, the difference of wettability is measured by a contact angle of solutions on polymer films. The contact angles of water and the acidic $PdCl_2$ aqueous solution on the hp film were $114 \pm 2°$ and $118 \pm 5°$, respectively, which indicates that the wettability was too low to be adsorbed the catalytic nuclei on the hp surface. On the other hand, the contact angle of the saturated ethanol solution of $PdCl_2$ on the hp film was less than 5°. Due to the high wettability between the hydrophobic hp surfaces and catalytic ethanol solution, homogeneous adsorption of the Pd catalytic nuclei was completed. It was found that wettability between template surfaces and catalytic solutions is very important for all-wet electroless plating.

3.2. Ni/P coating of the ps films

Fig. 4a shows an SEM image of the ps film having periodic pillars prepared by peeling off the top layer of the hp film. Contact angles of water and the acidic $PdCl_2$ aqueous solution on the ps film were $145 \pm 5°$ and $148 \pm 6°$, respectively, which means that the ps film possesses more hydrophobic surface than the hp film. This hydrophobic property was caused by the periodic pillar structures [4]. As is the case with the hp films, electroless Ni/P deposition after catalyzation by the acidic $PdCl_2$ aqueous solution does not proceed. Fig. 4b shows an SEM image of the Ni/P coated ps film by all-wet electroless deposition after immersion in the saturated ethanol solution of $PdCl_2$. Immersion in the saturated ethanol solution of $PdCl_2$ gave uniform adsorption of the catalytic nuclei on the hydrophobic surfaces of the ps films since the contact angle of the saturated ethanol solution of $PdCl_2$ on the ps film was under 5°. These results clearly indicate that immersion in the ethanol solution of $PdCl_2$ is suitable for catalyzation of hydrophobic surfaces for metal deposition by all-wet electroless plating.

3.3. Electroless plating in porous cavities of the hp films

It was found that inner surfaces of the hp film were covered by the amphiphilic copolymer **1** having carboxyl groups because temporarily formed water–solution interface in the formation process of the hp film was condensed by the amphiphilic copolymer **1** [10]. In the case of immersion in the acidic $PdCl_2$ aqueous solution, inhomogeneous metal deposition from the pore edges of the hp film was observed in Fig. 3c. Herein we hit on the idea of site-selective metal deposition by using the difference of wettability between inner and outer surfaces of the hp film. A

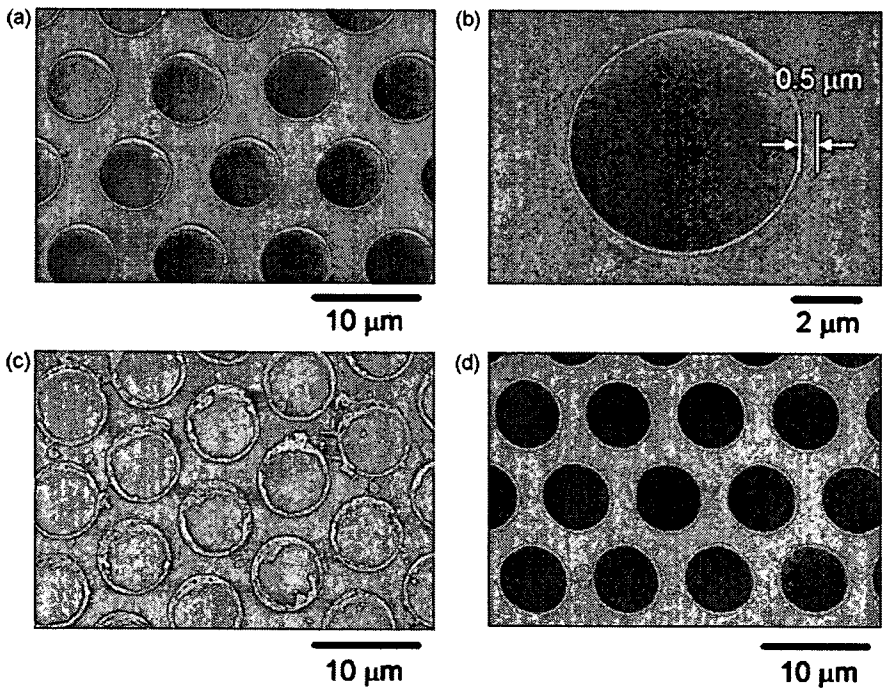

Fig. 3. SEM images of (a) the hp film, (b) its magnified image, (c) partly Ni/P-coated hp film by the all-wet electroless plating after immersion in the $PdCl_2$ acidic aqueous solution and (d) uniformly Ni/P-coated hp film by the all-wet electroless plating after immersion in the saturated ethanol solution of $PdCl_2$.

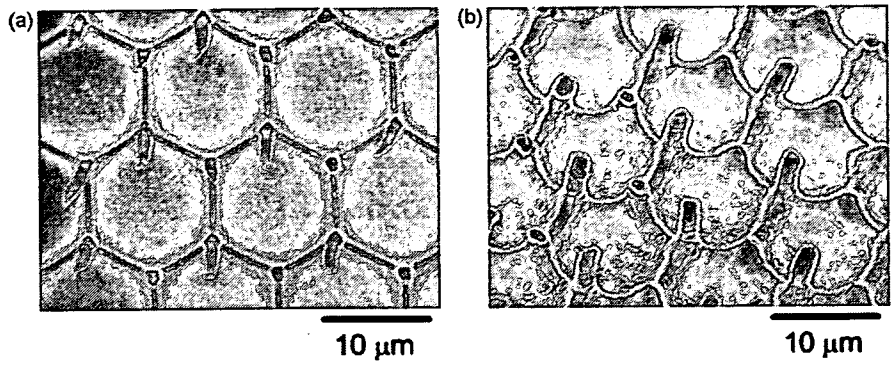

Fig. 4. SEM images of (a) the ps film and (b) Ni/P coated ps film by the all-wet electroless plating including immersion in the saturated ethanol solution of $PdCl_2$.

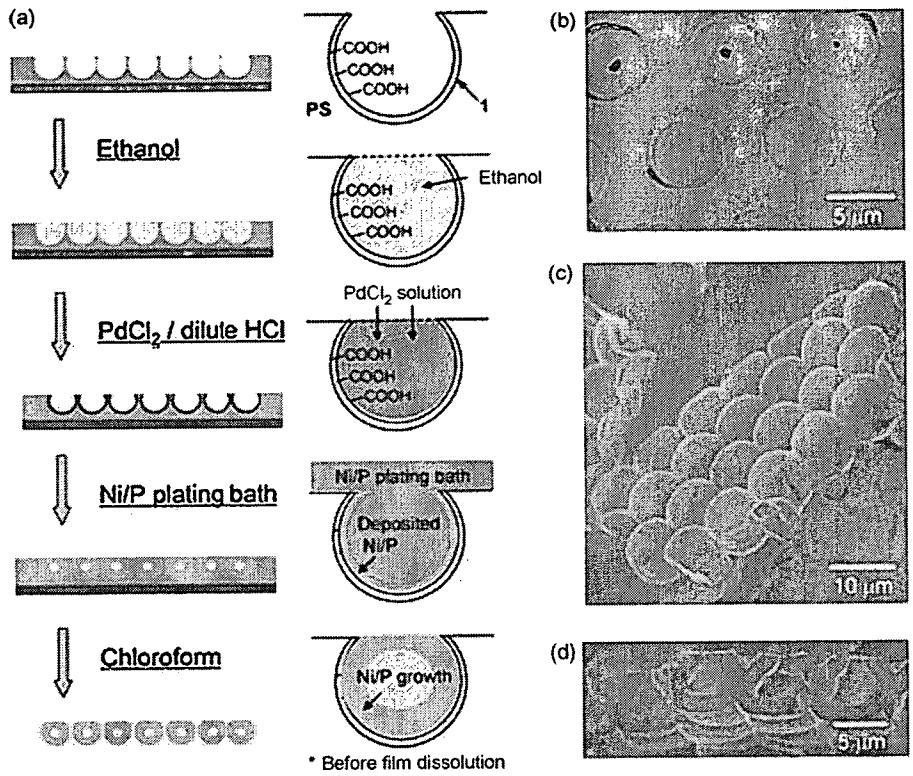

Fig. 5. (a) Schematic illustrations of site-selective metal deposition of the hp film by all-wet process, and SEM images of (b) the site-selective metal deposited hp film, (c) the metallic order structure after dissolution of the hp film, and (d) a cross section of the metallic order structure.

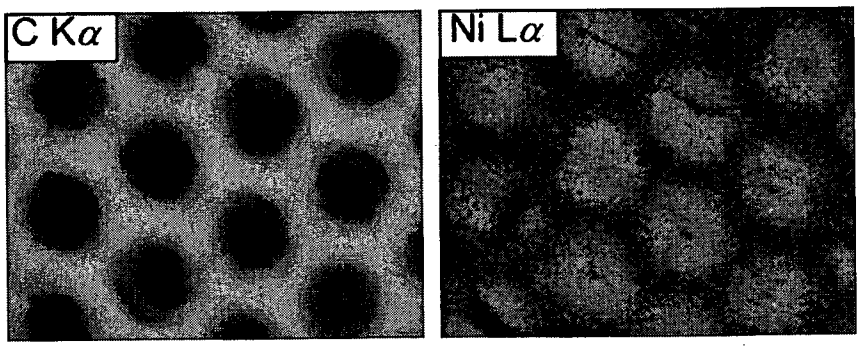

Fig. 6. EDX analysis of the site-selective Ni/P deposited hp film. Green dots in C Kα and red dots in Ni Lα indicate existence of a carbon atom of the hp film and of a deposited nickel atom by site-selective all-wet electroless plating, respectively.

schematic illustration of preparation method for metallic ordered structures by site-selective all-wet electroless metal deposition is depicted in Fig. 5a. Firstly, as pretreatment for catalyzation, porous cavities of the hp film were filled with ethanol having high wettability on the hp film. Then the pretreated film was immersed in the acidic $PdCl_2$ aqueous solution, after that immersed in the Ni/P electroless plating bath.

Fig. 5b indicates an SEM image of a site-selective Ni/P deposited hp film. Ni/P deposition was observed in only porous cavities of the hp film. An EDX mapping analysis as shown in Fig. 6 was performed in order to confirm the site-selectivity. Nickel was only observed in the porous cavities of the hp films, which means that site-selective deposition was brought about from inner surfaces of the hp film. A self-supporting Ni/P film composed of periodic metal spheres was obtained by dissolving of the template hp film with chloroform. Fig. 5c and d show SEM images of the ordered metallic structure and its cross sectional image, respectively. The ordered metallic structure was composed of conjunction of the hexagonally arrayed hollow spheres. The hollow spheres were formed by gradual metal deposition from inner surfaces of the hp film. These results clearly indicated that site-selective metal deposition of the hp film could be carried out by using the difference of wettability between inner and outer surfaces of the porous cavities.

4. Conclusion

In this report we demonstrated metal coating on hydrophobic surfaces of the polymer film having ordered structures by all-wet deposition process. Wettability of a catalytic solution on the substrate surface is very important for all-wet electroless plating. The saturated ethanol solution of $PdCl_2$ is best suited to catalytic solution of polymer films having hydrophobic surfaces by all-wet electroless plating. In addition, site-selective metal deposition of porous cavities of the hp film was carried out by using the difference of wettability between inner surfaces covered by amphiphilic copolymer **1** and outer surfaces mainly composed of polystyrene of the honeycomb structures. Physical properties of the ordered metallic structures are under investigation.

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