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Biosurface Design for Patterned Cell Culture Engineering

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Using poly(ethylene glycol) (PEG) possessing methacryloyl groups at both ends (PEG-DMA), patterned PEG gel surface was constructed on the silanized glass surface by photo-lithography technique. Prior to the photo-irradiation, the prepolymer coupled with photoinitiator (Irgacure 2959) was spin-coated on the silanized glass surface. Two casting solvents, methanol and methanol/water (50/50 vol. %) were employed because of low solubility of Irgacure 2959 in aqueous media. Using stainless mask having 100 μm aligned holes separated by 100 μm intervals (edge-to-edge), patterned circular PEG gels with 100 μm diameter on the glass surface was constructed by both casting solvents. When the pattern was constructed using methanol as casting solvent, endothelial cells were attached on the glass surface, but not on the circular PEG gel surfaces to obtained aligned endothelial cell patterned surface. On the contrary, when the pattern was constructed by water/methanol mixture as a casting solvent, endothelial cells were attached on the PEG gel surface, but not on the glass surface. Thus, completely reversed patterned culture was achieved only by changing casting solvents. Surface analyses of the patterned surfaces were carried out to understand the difference in these cell attachment phenomena.

Key word: Patterned cell culture, Photo-lithography, Endothelial cell, PEG patterned gel, Nega-posi pattern

INTRODUCTION

One of recent trends in life sciences, especially in pharmaceutical sciences, is to reduce avoidable animal experiments from the aspect of both cost and morality[1]. For example, numerous animal experiments have been carried out as a first screening of millions of compounds, which are synthesized, for example, by combinatorial chemistry technique[2]. In order to accomplish numerous drug screening in parallel, it is proposed to use an integrated microarray system using living cells, especially hepatocyte cells and tissues in stead of animal experiments[3]. We have so far reported that hepatocyte spheroids could be cultivated on patterned glass substrate, when Bovine aortic endothelial cells (BAECs) pattern was preconstruct as a feeder layer[4]. 100 μm circular domains of BAECs aliened at intervals of 100 μm were the most stable pattern as the feeder layer. The surface surrounding the domains were covered by dense PEG tethered chains. Cocultivation of hepatocytes with BAECs was essential to stabilize hepatocyte viability and liver-specific functions, allowing us to obtain hepatocyte spheroids with a diameter of 100 μm, functioning as a miniaturized liver to secret albumin for at least one month.

In order to control cell attachment on the surface, we have so far employed tethered PEG chains on the surface[5]. PEG tethering surface prevent not only proteins and lipids but also cells almost completely when the density of the PEG chains attained enough. The construction and patterning of the surface by plasma etching process, however, are time consuming and close attention is required for this process.

In this work, an alternative method using PEG hydrogel, which is commonly employed to avoid protein and cell attachment to the substrate surface, was examined[6]. If the PEG gel pattern shows the same effect as densely packed PEG tethered chains, the hepatocyte spheroid pattern can be constructed much easier and with high reproducibility. In addition, the hydrogel pattern may be useful for constructing functional cell arrays; *viz.*, the patterned gel can be used as a reservoir for functional compounds such as drugs and genes[7]. The functional compounds incorporated in the patterned gel may be transferred to the cultivated cells, which results in the creation of functional cells in a patterned array.

EXPERIMENTAL SECTION

Materials. Poly(ethylene glycol) (MW: 4,600), 3-(trimethoxysilyl)propyl methacrylate (TSPM) and 3-aminopropyltriethoxysilane (APTS) were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and were used as received. Commercial tetrahydrofuran (THF; Kanto Chemical, Tokyo Japan) and methacrylic anhydride (Aldrich) were purified by conventional methods[8]. A THF solution of potassium naphthalene was prepared by reacting naphthalene (13.4 g, 0.105 mol) with metallic potassium (4.30 g, 0.110 mol) in dry THF (300 mL). The mixture was allowed to react for overnight at room temperature, and the concentration of the mixture was determined by titration (0.35 mol/L). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich, and fetal bovine serum (FBS) and antibiotic-antimycotic were purchased from Gibco-Invitrogen (Grand Island, NY, USA). The water used in this study was purified using a Milli-Q system (Nihon Millipore Co., Tokyo, Japan).

Synthesis of methacryloyl-ended telechelic poly(ethylene glycol). To a THF solution (60 mL) of 1 mmol PEG with hydroxyl groups at both ends (MW; 4,600) in a 300 mL flask equipped with a three-way

stopcock, 2 mmol of potassium naphthalene and 6 mmol of methacrylic anhydride were added under a nitrogen atmosphere. After the mixture was allowed to react for several hours with magnetic stirring, the solution was poured into cold isopropyl alcohol (IPA) (1.5 L). The precipitate was centrifuged and the collected precipitate was dissolved in THF (50 mL). The solution was poured again into cold IPA (1.5 L). The precipitated polymer thus obtained was finally freeze-dried with benzene (yield: 98%). The obtained polymer was analyzed by size exclusion chromatography (SEC) and ^{1}H NMR spectra, and an end-functionality of approximately 90 % was confirmed.

Construction of a PEG gel micro-patterned surface. After the glass surface was washed with piranha solution (1:1 volume of concentrated H_2SO_4 and hydrogen peroxide (30 w/v%) for 1 h), the surface was modified with an ethanol solution of 3-(trimethoxysilyl)propyl methacrylate (1.6 vol.%) for 30 min and gently rinsed with ethanol, followed by treatment with 3-aminopropyl-trimethoxysilane (4 vol%). Then, the glass was washed with deionized water three times and allowed to stand for 12 h at 100 °C in vacuo. The mixture of PEG-DMA (MW: 4,600; 50 mg) and the same amount of Irgacure 2959 in methanol (1 mL) (Method A) and/or in a water/methanol cosolvent (50 % vol/vol, 1 mL) (Method B) were spin-coated at 3,000 rpm on the silanized glass surface. After the casting solvent was removed completely by evaporation at ambient temperature for 10 min, the micro-pattern was prepared by UV exposure (254 nm, 240 mJ/cm^2). A metal mask with 100 μm aligned cavities separated by 100 μm intervals (edge-to-edge distance) was used for patterning through the metal mask under a narrow bandwidth. The surface was developed by distilled water for 30 s to remove unexposed areas of PEG-DMA film. The obtained micro-patterned surface was soaked in PBS for 30 min prior to use.

Endothelial-cell culture on PEG patterned gel surfaces. Bovine aortic endothelial cells (BAECs) were purchased from the Health Science Research Resources Bank (JCRB0099, Osaka, Japan). BAECs under 20 passages were used in all cell culture experiments. All modified glass substrates were sterilized in 70 % ethanol and then immersed in DMEM supplemented with 10 % FBS and 1 % antibiotic-antimycotic for 30 min at room temperature. 2.0 x 10^5 cells/cm^2 BAECs, which is the same amount of confluent cell adhesion on the surface used, were seeded onto patterned PEG gel glass substrates and incubated at 37 °C with DMEM in a humidified atmosphere with 5% CO_2. After 24 h of cultivation at 37 °C, the unattached cells were washed away with PBS. The cell morphology was monitored using a phase-contrast microscope (OLYMPUS IX71, OLYMPUS Co., Tokyo, Japan).

Protein adsorption experiments. For these experiments, we prepared five model surfaces, as follows: After methanol solutions containing both PEG-DMA and photoinitiator were spin-coated on silanized glasses, the surfaces were developed by water before (Glass A) and after (Gel A) photo irradiation. Alternative samples were obtained using methanol/water as co-solvent for spin coatings (Glass B and Gel B). In addition, silanized glass, which was not treated with PEG solution, was prepared as a control. A fluorescein isothiocyanate conjugated bovine serum albumin (FITC-BSA) adsorption test was carried out on the patterned PEG hydrogel surface by soaking each substrate in PBS containing FITC-BSA (1 mg/mL) for 90 min at room temperature after preincubation in PBS. Subsequently, the samples were rinsed three times with PBS for 10 min. The adsorbed protein on the surface was analyzed using a fluorescence plate reader (ARVO™ MX, PerkinElmer Japan Co., Ltd., Yokohama, Japan) and a fluorescence microscope (OLYMPUS IX71, OLYMPUS Co., Tokyo, Japan). The fluorescence intensity was measured at 485 nm excitation and 535 nm emission.

Contact angle of the PEG-treated surface. The contact angles of water on the prepared silanized glass and PEG gel surfaces were measured using a contact angle analyzer (CA-X, Kyowa Interface Science Co., Ltd., Asaka, Japan) as follows: a liquid droplet was gently placed onto the surface, and the contact angles were measured 10 s after placement by the θ/2 method.

XPS analysis. The chemical composition of the surface was determined by X-ray photoelectron spectroscopy (XPS) using a magnesium anode nonmonochromatic source (AXIS-Hsi, Shimadzu/KRATOS ANALYTICAL, Ltd., Kyoto, Japan). All samples were completely dried in vacuo before measurement. Survey scans (0-1100 eV) were performed to identify the C and Si elements. The takeoff angle of the photoelectrons was 90°. All the binding energies referenced the C1s peak at 285.0 eV. Their elemental compositions were determined based on the peak areas corresponding to these elements.

RESULTS AND DISCUSSION

Construction of PEG gel patterned surface

In order to fabricate cell-patterned surfaces, the attachment of cells on the substrate surface must be precisely controlled; i.e., the cytophilic and cytophobic surfaces must be precisely constructed. Because hydrogel surfaces are well known to reduce cell adhesion, we employed PEG hydrogel as a cytophobic surface. If a PEG gel pattern can be precisely constructed on a silanized hydrophobic glass surface, the cell pattern should be formed easily. Photolithography can be utilized for the preparation of such patterned surfaces. Thus, we employed photolithography for the construction of patterned PEG hydrogel layers on a glass surface. The obtained telechelic PEG, coupled with Irgacure 2959 as a photoinitiator, was used as the patterning material. Though Irgacure 2959 is reported to be biologically benign[9], it is hardly soluble in pure water. Thus, the prepolymer coupled with Irgacure 2959 was dissolved in methanol (Method A) and/or methanol/water co-solvent (Method B) and spin-coated on the silanized glass substrate.

The gel patterns were observed by microscopic analysis. From the microscopic observation, almost the same patterns as the stainless mask used in this study was observed (data not shown). Thus, it is confirmed that a precisely controlled PEG gel micropattern on a hydrophobic glass surface could be obtained by using either of the spin-coated films (Methods A and B).

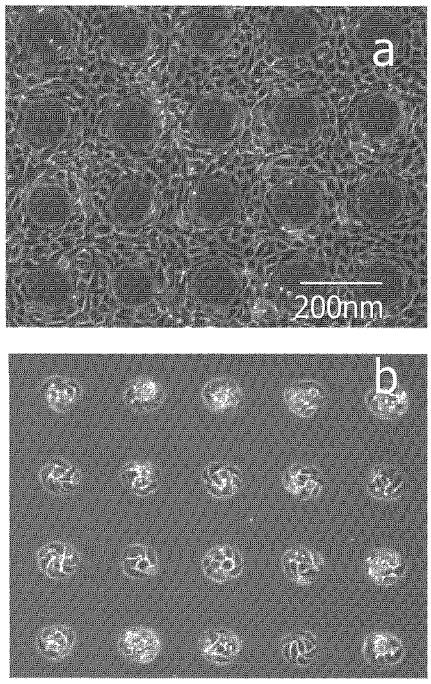

Figure 1. Phase-contrast micrographs of the inverse pattern of BAECs. BAECs cultured on a PEG gel pattern surface prepared by (a) Method A and (b) Method B. These surfaces were constructed using the same materials and photomask, except for the solvent in the polymer solution during the casting process.

Endothelial-cell culture on PEG patterned gel surfaces. In order to construct two-dimensional cell microarrays, BAECs were seeded on patterned PEG hydrogel substrates prepared by Methods A and B. When the prepolymer cast film was prepared using methanol solution (Method A), the BAECs adsorbed onto the silanized glass surface area but not on the PEG gel dot area. Consequently, a clear BAEC patterned surface aligned with the pattern could be obtained, as shown in Figure 1a. The PEG gel surface worked as the cytophobic area, as anticipated in this case. On the contrary, a completely reversed pattern of BAECs was observed when the BAECs were cultured on patterned surfaces prepared by Method B. In this case, the BAECs attached to the PEG gel surface but not to the glass surface. It should be noted that the only difference between these two patterned surfaces was the casting solvent involved. In addition, these casting solvents should be completely evaporated before the photo-crosslinking reaction. As mentioned above, it is well known that a surface coated with PEG hydrogel generally induces the reduction of the non-specific interaction of biomolecules such as proteins and cells on the surface of the substrate. The observed cell attachment on the surfaces prepared by Method B was totally opposite in character. Why does BAECs adsorb on the PEG dot surface prepared using methanol/water co-solvent, but not that prepared using pure methanol solvent? Why do BAECs not adsorb on bare glass surfaces produced by Method B? In order to obtain information on this peculiar phenomenon, i.e., the complete reverse cell adhesion not only on the PEG gel surfaces but also on the glass substrate surfaces, several surface characterizations were carried out in detail.

Protein adsorption. Because it is known that proteins tend to adsorb on cytophilic surfaces prior to cell adhesion, protein adsorption experiments were carried out using model surfaces. The amount of FITC-BSA adsorption on several model surfaces was monitored by a fluorescent plate reader. The data are summarized in Table 1. When the PEG gel was prepared using methanol solvent (Gel A), the adsorption of FITC-BSA was suppressed effectively, as expected. On the contrary, almost three times larger amount of FITC-BSA than that for Gel A, was adsorbed on the PEG gel surface prepared using methanol/water co-solvent (Gel B). The obtained data agreed well with the cell adhesion data. Cell attachment occurred after the adsorption of serum proteins on the PEG gel surfaces prepared using methanol/water co-solvent (Method B). Protein adsorption experiments were then carried out on the glass surfaces. Prior to the protein adsorption tests on the silanized glass, the glass surface was treated with PEG/Irgacure 2959 solutions (methanol and/or methanol/water), followed by washing with water, without photoirradiation. The obtained glass surfaces were evaluated. Based on the data obtained from the protein adsorption experiment, it is concluded that protein tends to adsorb on the glass surface rather than the gel surface when the gel is prepared using methanol solvent, while protein adsorbs on the gel surface rather than the glass surface when the gel is prepared using methanol/water co-solvent.

Contact angle measurements. In order to obtain further information on the surface characteristics, contact angle measurements were carried out, since it is widely recognized that an increase in surface hydrophobicity tends to promote stronger protein adsorption from the solution. From the contact angle measurements, it was found that the surface of Gel B prepared using methanol/water co-solvent was more hydrophobic than that of Gel A prepared using methanol solvent, as shown in Table 1. This was probably due to the difference in the distribution of initiator fragments in the gel matrix. It is known that cells tend to adhere well on surfaces with a contact angle of approximately 70 degrees [10]. The hydrophobic nature of the PEG gel (Gel B) caused by the methanol/water co-solvent increased the serum protein adsorption and consequently induced the adhesion of the BAECs.

The next question concerned the glass surfaces. During the photolithographic process, the glass surfaces were coated with polymer coupled with the initiator Irgacure 2959, followed by development with water. No photo-crosslinking was done. In order to confirm the differences between these surfaces, Glasses A and B were prepared as model surfaces and their surface contact angles were measured. When only casting, drying and developing were applied without photoirradiation, the contact angle of the surfaces was clearly different. When methanol was used, the contact angle was almost the same as that of silanized glass. When methanol/water co-solvent was used, the contact angle of the glass surface (Glass B) was fairly low, indicating the adsorption of PEG-DMA on the surface of Glass B. Haward et al. have reported that the adsorption of PEG on a glass surface was affected by the medium[11]. It is thus confirmed that the hydrophilic Glass B surface resulting from PEG-DMA adsorption has good

biofouling resistance, and consequently suppresses the attachment of BAECs.

XPS analysis of PEG adsorption on silanized glass surfaces. From the analyses described above, it is concluded that the surfaces prepared in this study were totally different (Methods A and B). Even for the glass areas, there are clear differences, even though the surfaces were merely spin-coated and developed without photoexposure. Their contact angles were fairly different. This is probably due to the difference in the amounts of PEG adsorption on the silanized glass according to the solvent used. Using XPS analysis, it is confirmed again the adsorption of PEG on the glass surface. The glass surfaces, which were spin-coated with different PEG solutions (Glass A: methanol; Glass B: methanol/water; Glass C: water), showed an alternative peak at around 286.5 eV, which is assignable to C-O peak[12]. With increasing hydrophilicity of the solvent (methanol → methanol/water → water), the peak intensity increased, confirming beyond doubt that PEG was adsorbed on the glass surface even after the development without UV exposure.

Table 1. Surface characterizations of PEG gel and glass prepared in this study

Sample	Protein adsorption (%[a])	Contact angle (deg)	C-O/Si[b]
Glass	-	72.2 ± 1.8	0.05
Gel A	25.6 ± 8.9	36.5 ± 2.5	-
Gel B	65.7 ± 8.4	62.6 ± 1.1	-
Glass A	80.1 ± 25.9	60.9 ± 3.9	0.65
Glass B	31.1 ± 9.6	35.6 ± 1.5	1.05
Glass C[c]			1.56

[a]Amount of FITC-BSA adsorption relative to silanized glass surface was determined in percentage. [b]Determined by XPS data. [c]Glass C was prepared by spin coating of PEG/Irgacure in pure water, followed by development without photo-irradiation

CONCLUSIONS

In summary, we have demonstrated the construction of PEG hydrogel patterned surfaces using photolithography. During the preparation of the patterned surfaces, we found that complete reverse cell adhesion patterns were formed by changing one parameter: the solvent used in film casting during the preparation of the gel patterns. From the protein adsorption, contact angle and XPS analysis data, it is clear that Methods A and B yielded quite different PEG gel surfaces and glass surfaces. When methanol was employed as the casting solvent, the PEG gel areas on the patterned surface showed hydrophilicity and prevented cell adsorption, while the silanized glass areas showed hydrophobicity, allowing the adsorption of BAECs. On the contrary, when methanol/water was used as the casting solvent, the adsorption tendency was totally opposite. The difference in the PEG gel surfaces is probably due to the distribution of the initiator fragment, because of its poor solubility in aqueous media. The difference in the adsorption characteristics of PEG from that of the solvent used for the glass surface is the main reason for the difference in cell adsorption on the glass area. By changing the casting solvent for the preparation of the PEG gel pattern in this way, complete negative and positive patterns can be constructed on a glass surface using the same substances and the same mask. This technique may widen the scope of cell patterning methodology.

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