Construction of Spheroid Cell Microarray on Patterned PEG Hydrogel Surface

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1. Introduction

Control of cell attachment and its functionality on the substrate surface is one of the most important subjects in versatile fields such as in vitro bioassay, tissue engineering, etc. [1-2]. Among in vitro cell culture techniques, micro-patterned cell and tissue culture have recently attracted growing attention as a high-throughput bioassay system, because of their facile clinical and pharmaceutical analysis of molecular targets, which avoid certain amount of animal experiments [3-6]. Especially, hepatocyte cell array culture is important for drug screening and clinical pharmacology study. Conventional primary hepatocyte cell culture in a two dimensional plate, however, loses its metabolic functionality easily within few days. A spheroid culture has attracted attention as a three dimensional cultivation technique, which retains their metabolic functionality for a long term. There are several methods to prepare three dimensional primary hepatocyte spheroid. For example, spinner cultures [7], cultivation on porous film [8], use of non-adherent cell culture plate [9-10].

Recently, we have reported hepatocyte spheroids array on the patterned PEG tethered chain glass surface [11]. A two-dimensional microarray of hepatocyte spheroids, underlaid with endothelial cells, was successfully constructed with 100 μm spacing on microfabricated glass substrates that were coated with PEG tethered chains. Cocultivation of hepatocytes with endothelial cells 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 secrete albumin for at least one month. To construct patterned PEG tethered chain surface, plasma etching process was

employed. This method is effective to construct extremely clear micropatterning surface, though it takes fairly long time. This paper communicates facile construction of patterned surface using PEG micro gel via photo-lithography technique. Construction of heterospheroid array and its functionality using established cell (FLC-4) on the endothelial cell pattern are described.

2. Method

2.1. Synthesis of Methacrylate-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 potassium naphthalene and 6 mmol methacrylic anhydride were added under nitrogen atmosphere. After the mixture was allowed to react for 24 h with magnetic stirring, the solution was poured into isopropyl alcohol (IPA). The precipitates were centrifuged to collect and precipitated again from THF solution. The polymer thus obtained was finally freeze dried with benzene. The obtained polymer was analyzed by GPC and ^1H NMR spectra and ca. 90 % of end-functionality was confirmed (data not shown).

2.2. Construction of PEG Gel Micro-patterning Surface

After the glass surface was washed by Piranha solution (1:1 volume ratio of conc. H_2SO_4 and hydrogen peroxide (30 w/v%) for 1 h), the surface was modified with ethanol solution of 3-(trimethoxypropyl) methacrylate (2 wt%) for 30 min, followed by the treatment with 3-aminopropyltrimethoxysilane (4 vol.%). Then the glass was washed with deionized water and reacted

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at 100 °C for 12h. The mixture of PEG-DMA and Irgacure 2959 (5wt%) in water/methanol cosolvent (1:1) was spin coated at 3,000 rpm on the silanized glass surface. The micro-pattern was prepared by the irradiation of UV light with patterned mask (240mJ/cm²). The surface was developed by distilled water (Figure 1). The obtained micro-patterned surface was soaked in PBS for 30 min prior to use.

Fig. 1. Process flow for the fabrication of PEG hydrogel microstructures.

2.3. Protein Adsorption Experiments

FITC conjugated BSA adsorption test on the patterned PEG hydrogel surface was carried out by soaking the substrate in 1 mg/mL of FITC conjugated BSA in PBS for 90 min at room temperature. The adsorbed protein was analyzed by fluorescence microscopy (OLYMPUS IX71).

2.4. Patterned Cell Culture of FLC-4 Cell and Endothelial Cells (ECs)

Bovine aortic endothelial cells (BAECs) were purchased from 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 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic for 30 min at room temperature. The amount of equalized BAECs as area of a substrate were seeded onto patterned PEG gel glass substrates and incubated at 37 °C with a DMEM in a humidified atmosphere with 5% CO₂. After 24 h of culture at 37 °C, unattached cells were washed away with the PBS. Hepatic cancer cells (FLC-4) (supplied from BIOS Inc.) were seeded

into these dishes at the cell density of 7×10^5 cells per 3.8 cm² and cultured at 37 °C with a DMEM/F12 supplemented with 10% FBS, 1% penicillin and streptomycin in a humidified atmosphere with 5% CO₂. Cell morphology was monitored by a phase-contrast microscope (IX71, OLYMPUS JAPAN).

2.5. Measurement of Hepatocytes Function

Albumin secretion by FLC-4 cells in spheroids underlaid with endothelial cells and monolayer culture was determined by an immunohistochemical double-staining method and sandwich enzyme-linked immunosorbent assay (ELISA). For fluorescence microscopy observation, cultured cells in spheroids and monolayer for 24h were fixed at 37 °C with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed surfaces were washed with PBS and permeabilized with acetone at -20 °C in PBS for 5 min and then rinsed with PBS twice. Surfaces were then blocked with 1% BSA in PBS for 30 min, and incubated with 10 µg/mL goat anti-human albumin antibody and 1% BSA in PBS overnight at 4 °C. Following washes with PBS several times, the surfaces were incubated for 30 min with a Alexa Fluor 488 rabbit anti-goat IgG, Alexa Fluor 555 phalloidin and 1% BSA at room temperature. The stained surfaces were mounted and observed under a microscope with fluorescence equipment (Alexa488 ; Abs. 495nm / Em. 519nm, Alexa555 ; Abs. 555 / Em.565nm) after plenty of washing with PBS.

Measurement of a time-lapse albumin secretion by ELISA of FLC-4 spheroids, underlaid with endothelial cells and FLC-4 monolayer were carried out by using anti-human albumin antibody. HRP-conjugated sheep anti-human albumin was used as a secondary antibody. LmiGLO(KPL) was employed as a chemiluminescent substrate of horseradish peroxidase. The intensity of chemiluminescence was measured with a microplate reader. Living cell number was determined by the LIVE/DEAD cell vitality assay kit (Molecular Probes) after isolation of FLC-4 from spheroid with 0.1% trypsin.

3. Results

3.1. Characterization of Patterned PEG Gel Surface.

The objective of this work was to construct patterned PEG hydrogel on the glass surface by photolithography technique. In order to confirm the patterned PEG gel on the surface, protein and cell adhesion tests were carried out using

fluorescent-labeled BSA and endothelial cell. Fig. 2(a) shows fluorescence image on patterned PEG gel surface after incubation with PBS solution of FITC-BSA. The white area demonstrates a fluorescent signal based on FITC-BSA, which corresponds to the mask pattern employed in this study. The fact means that the FITC-BSA adsorbed on the PEG gel surface selectively, but not on the glass surface. Fig. 2(b) shows the phase contrast microscope results of the BAECs culture on the patterned surface after cultivation for 24 h. As can be seen in the figure, the cell was adsorbed on the same area as that of BSA, indicating that the patterned PEG gel surface shows protein and cell adsorption tendency. In general, PEG gel surface tends to suppress the protein and cell adsorption [12]. Our surface, however, was totally opposite results, which will be analyzed and published elsewhere.

Fig. 2 BSA and BAEC adhesion on patterned PEG gel surface. (a) Fluorescent micrograph of FITC-labeled BSA and (b) phase contrast micrograph of BAECs.

3.2. Spheroid Culture

We have already reported that primary hepatocyte cells can be cultured on the

Fig. 3 FLC-4 cell cultivation on the PEG patterned gel surface for 1 week. (a) spheroid cultivation pattern (x40), (b) The are of two spheroids fusion (x40), (c) magnification of spheroid (x200)

preconstructed patterned endothelial array as a spheroid shape. In order to extend these spheroid patterning culture system, established cell (FLC-4: human hepatic cancer cell) as a spheroid source was examined. When FLC-4 was incubated directly on the PEG gel patterned surface, cell spheroid array was easily obtained as shown in Figure 3a, though some of spheroids were over-grown to merge pattern as shown in Figure 3b. It is interesting to note that the height of the spheroid increased as a function of time and resulted in circular cone as shown in Figure 3c. The number of the cell in each array was roughly estimated to 100. After the endothelial cell patterning was constructed on the surface, the equal amount of FLC-4 cells / surface area was seeded. As shown in Fig. 4a, FLC-4 spheroid was constructed on the BAEC spherical patterns. After the one week cultivation, however, FLC-4 was over-grown to form monolayer pattern, indicating the fast growing of FLC-4 cells under the present conditions (Figure 4b)

Fig. 4 Overgrowth of FLC-4 spheroids with underlaid BAECs (a) cultivation for 24h and (b) for 1 week.

3.3. Analysis of Specific Function of FLC-4 Spheroid Array

In order to confirm specific function of FLC-4 cells as a spheroid pattern, albumin secretion as a function of cultivation time was evaluated. Fig. 5 shows fluorescent microscopy of monolayer and patterned spheroid FLC-4 cell array. In the case of FLC-4 monolayer culture, F-actin can be stained clearly (Figure 5b), while albumin did not stained well (Figure 5c). On the contrary, both F-actin and albumin were stained clearly in the case of FLC-4 spheroid cell array culture. The quantitative results of the albumin secretion were determined by the ELISA method. As shown in Fig. 6, albumin secretion was ca. 5 pg/cell after one day and it is almost the same up to 4d cultivation in the case of monolayer culture. On the contrary, albumin secretion for one day from the spheroid pattern on the endothelial feeder layer was more than 4 times higher than that of the monolayer cultivation. In addition, the albumin secretion from the FLC-4

Fig.5 Immunohistochemical double-stained FLC-4 monolayer cultivation [(a)-(d)] and spheroid cultivation [(e)-(h)]. (a) and (e) are taken by phase contrast microscopy. (b) and (f) are F-actin staining, (c) and (g) are albumin staining. (d) and (h) are superimposed images.

spheroid array was increased up to 4 days. On the basis of these results, FLC-4 spheroid array on the endothelial feeder layer was high cell functionality as compared to monolayer cultivation and is promising as a useful cell patterning tool.

4. References

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Fig. 6 Time course of albumin secretion from FLC-4 monolayer cultivation (square) and spheroid cultivation (circle).

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