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Inverted Pattern Formation of Cell Microarrays on Poly(ethylen glycol) (PEG) Gel Patterned Surface and Construction of Hepatocyte Spheroids on Unmodified PEG Gel Microdomains†

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We present herein the novel technique for constructing inverted cell-adhesion patternes on PEG gel modified glass surfaces by photoirradiation using same photomask and materials. The PEG gel micropatterns were prepared by photolithographic technique using a photomask with 100 μm aligned cavities after spin-coating of a mixed solution of •,• -dimethacryloyl-PEG (PEG-DMA) and a photoinitiator on glass surfaces. When methanol was used as a casting solvent for the spin-coating (Method A), the circular PEG gel domains with a diameter of 100 μm were fabricated on the surface, and as would be predicted, seeded bovine aortic endothelial cells (BAECs) adhered to the glass area on the constructed surface to form a BAECs sheet with 100 μm aligned cavity. In contrast, it was rather surprising for us that a complete inverted cell pattern was formed when the PEG gel pattern surface was prepared using methanol/water co-solvent (Method B). Furthermore, when hepatoma cancer cells were seeded on the constructed surface prepared by Method B, they formed a spherical multicellular aggregate (spheroid) on the unmodified PEG gel domains without feeder cells. In order to obtain information on this peculiar phenomenon, fluorescence-based protein adsorption experiments, contact angle measurements, and X-ray photospectroscopy (XPS) analysis were carried out.

Introduction

Recent progress in cell culture and microfabrication technologies has stimulated researches on the integration of cell cultures and sensors on a chip. New high-throughput techniques based on cells and tissues microarrays will not only contribute to understand fundamental cell biology but also facilitate clinical and pharmaceutical analysis of molecular targets, because living cells can monitor the targets through the physiological changes that are induced in them by exposure to drugs and environmental perturbations, such as toxicants, pathogens or other agents[1-4].

Primary hepatocyte are the most useful candidates for constructing cell- and tissue-based biosensors, because hepatocyte play many roles in drug metabolism in vivo. However, primary hepatocyte rapidly lose their liver-specific functions under conventional two-dimensional cell culture conditions. Interestingly, we have recently reported that hepatocyte spheroids formation can be facilitated on patterned glass substrates, where the surface surrounding the domain was covered by dense PEG tethered chains and the bovine aortic endothelial cells (BAECs) pattern is preconstructed as a feeder layer on the glass surface[5, 6]. Multicellular spheroids exhibit a characteristic in vivo-like morphology and cellular environment that can be used to determine gene expression and the biological behavior of cells[7-9]; this is attributed to the retention of the 3-D architecture and establishment of important cell–cell contacts[10]. Thus, tumor spheroids have served as models for a variety of experimental studies. Alternatively, cell-based bioassays that use primary hepatocyte act as attractive and important methods for studying the specific functions of the liver. In the cell microarray based on the PEG patterned technique, the density of the PEG tethered chains forming the cell-incompatible surface plays a key role to maintain the long-term cultivation and albumin secretion of hepatocyte spheroid. If the density of the PEG chains on the surface is not sufficiently high, the BAECs in the patterned domain start to overgrow easily, resulting in the collapse of micro-patterned formation of hepatocyte spheroids. For the construction of a densely packed PEG brush pattern surface, the surface coating must be done carefully using precisely controlled PEG block copolymer. We have so far employed a plasma etching process to construct surfaces with precise patterns, but this process requires the increase of the number of processing steps under high vacuum conditions.

In this work, an alternative method for constructing cell microarray using PEG hydrogel, which is commonly employed to avoid protein and cell attachment to the substrate surface, was examined[11-14]. If the PEG gel pattern shows the same effect as densely packed PEG tethered chains, the hepatocyte spheroid pattern can be constructed much more easily and with high reproducibility. Interestingly, during the preparation of the patterned surface, we found that the complete inverted cell adhesion patterns of BAECs were formed as a result of slight differences in the preparation conditions of the gel patterns: the casting solvent used in the film formation step during the gel patterns preparation. Furthermore, spheroid formation of human hepatoma cancer cells (FLC-4) on the unmodified PEG-gel microdomains without feeder cells was also observed. In order to obtain information on this peculiar phenomenon, fluorescence-based protein adsorption experiments, contact angle measurements, and XPS analysis were carried out.

Results and Discussion

Fabrication of PEG-gel patterned microarray on glass surface and formation of inverted micro-patterned of BAECs

In order to fabricate cell-patterned surfaces, the cell-compatible and cell-incompatible surfaces must be precisely constructed. Because hydrogel surfaces are well known to reduce cell adhesion, we employed PEG hydrogel as a cell-incompatible surface. Photolithography method can be utilized for the preparation of PEG gel patterned microdomains on a silanized hydrophobic glass surface. PEG with a methacrylate group at both ends, coupled with Irgacure 2959 as a photoinitiator, was used as the pattering material. Irgacure 2959 is reported to be biologically nontoxic[15] and it is hardly soluble in pure water. Thus, the prepolymer coupled with Irgacure 2959 was dissolved in methanol and/or methanol/water co-solvent and spin-coated on the silanized glass substrate. The constructed two PEG-gel patterned surfaces were analyzed by differential interference contrast (DIC) microscope analysis and the fomraiton of PEG-gel microdomains on the glass surfaces were confirmed (Figure S4, see ESI).

In order to construct two-dimensional cell microarrays, BAECs were seeded onto the constructed PEG hydrogel patterned substrates. As shown in Figure 1a, when circular PEG gel domains with a 100 μm diameter were fabricated by the UV irradiation of PEG-DMA film prepared by casting using methanol (Method A), BAECs adhered to the glass area on the constructed surface to form a BAECs sheet having 100 μm aligned cavities. The PEG gel domain surfaces worked as the cell-incompatible area, as anticipated in this case. Surprisingly, a complete inverted cell array pattern was formed when the pattern was prepared using film cast from methanol/water co-solvent (Method B), as shown in Figure 1b. In this case, the BAECs adhered to the PEG gel domain circles selectively avoiding the glass area on the constructed surface. It should be noted that the only difference between these two patterned surfaces was the casting solvent involved, which should be completely evaporated before the photocrosslinking reaction.

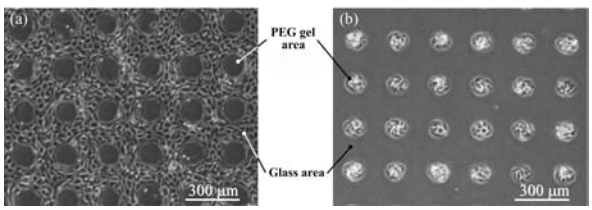

Fig. 1 Phase-contrast micrographs of the inverse pattern of BAECs. BAECs cultured on a PEG gel patterned surfaces prepared by (a) Method A and (b) Method B. These surfaces were constructed using the same materials and photomask, except for the solvent in the polymer solution during the casting process.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

Comparison of the adsorption amount of protein on the PEG gel patterned surfaces and model surfaces

Protein adsorption experiments were carried out on the constructed PEG gel patterned surfaces and model surfaces. Figure 2a shows fluorescence microscopy images of the PEG gel patterned surfaces treated with FITC-BSA. As shown in the images, clear contrasts were observed: a larger amount of protein adsorbed on the silanized glass area in PEG gel patterned surface prepared by Method A (Figure 2a left), while the protein adsorbed on the PEG gel area in the surface prepared by Method B (Figure 2a right). Additionally, FITC-BSA adsorption experiments were demonstrated on the model surfaces, Gel A, Gel B, Glass A and Glass B surfaces, which were prepared by the treatment methods summarized in Table 1. Figure 2b shows the amount of FITC-BSA adsorption on the model surfaces measured by fluorescent plate reader. When the PEG gel was prepared using methanol solvent (Gel A), the adsorption of FITC-BSA was suppressed effectively, as expected. On the contrary, a large amount of FITC-BSA, more than three times higher 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 in Figure 1. 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 (Glass A: prepared using methanol solvent; Glass B: prepared using methanol/water co-solvent; see the Experimental section and Table 1). A large amount of FITC-BSA, more than two times higher than that for Glass B, was adsorbed on the Glass A surface. Furthermore, in the case of the surfaces prepared by methanol (Gel A and Glass A surface), larger protein adsorption was obserbed on the glass surface, while larger protein adsorption was obserbed on the gel surface in the case of the surfaces prepared by methanol/water co-solvent (Gel B and Glass B surfaces). As a conclusion, in the case of the PEG gel-patterned surface prepared by methanol, protein tends to adsorb on the glass surface area rather than the PEG gel microdomaines. On the other hand, when the gel-patterned surface was prepared by methanol/water co-solvent, protein adsorbs on the PEG gel microdomaines rather than the glass area.

Table 1 The treatment methods for each model sample

Samples	Treatment method (casting solvent of PEG with initiator in spin-coat)	UV irradiation
Silanized glass	-[a]	-
Gel A	Method A (methanol)	+
Gel B	Method B (water/methanol co-solvent[b])	+
Glass A	Method A (methanol)	-
Glass B	Method B (water/methanol co-solvent[b])	-

[a] No treatment with PEG and initiator solution. [b]50 % v/v. The difference between Method A and Method B was only casting solvent (See ESI).

Contact angle measurements on model surfaces

Since it is widely recognized that an increase in surface hydrophobicity tends to promote stronger protein adsorption from the solution, we investigated the variation of the contact angle on the model surfaces. Figure 2c shows the static water contact angles on the model gel surfaces (Gel A and Gel B, see Table 1) and the model glass surfaces (Glass A and Glass B, see Table 1).

It was found that the surface on Gel B (prepared by methanol/water co-solvent as a casting solvent) was more

hydrophobic (CA = 60°) than that of Gel A (prepared by methanol as a casting solvent) (CA = 35°). It is known that cells tend to adhere well on surfaces with a contact angle of approximately 70 degrees16. The serum protein adsorption on the Gel B surface was due to the hydrophobic nature of PEG gel, and consequently, the adhesion of the BAECs was also induced on the PEG gel microdomains on the patterned surface.

The next question concerns the difference in the amount of protein adsorption on the glass surfaces prepared by Method A and Method B. During the photolithographic process, the glass surfaces were coated with polymer coupled with the initiator Irgacure 2959, followed by development with water. The Glass A (prepared by methanol as a casting solvent) and the Glass B (prepared by methanol/water co-solvent as a casting solvent) were constructed by only casting, drying and developing with water were applied without photoirradiation (Table 1). The contact angles of these glass surfaces were clearly different. In the case of Glass A, the contact angle was almost the same as that of silanized glass (CA = 60°). In contrast, in the case of Glass B, the contact angle was fairly low (CA = 30°), indicating the adsorption of PEG-DMA on the Glass B surface. Haward et al. have reported that the adsorption of PEG on a glass surface was affected by the medium[17]. For example, a larger amount of PEG adsorbed on silica made using water solution than on silica made using methanol. The phenomena observed in this study are in good agreement with their results.

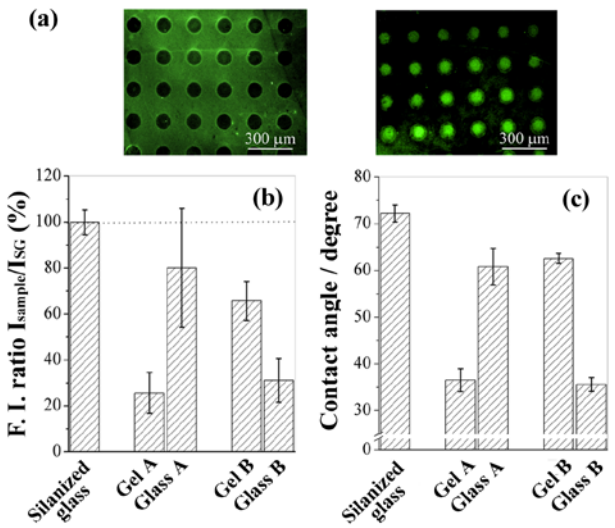

Fig. 2 Surface characterizations of PEG gel patterned surfaces and model surfaces by fluorescence-based analysis and contact angle measurements. (a) Fluorescence micrographs of FITC-BSA adsorbed onto PEG gel patterned surfaces prepared by Method A (left) and□ Method B (right). (b) Normalized fluorescence intensity on each model surface adsorbed by FITC-BSA. I_{sample} and I_{SG} are fluorescence intensity of the model surface and that of silanized glass surface, respectively. (c) Static water contact angles on several model surfaces.

XPS analysis of PEG adsorption on silanized glass surfaces

To obtain further quantitative information of PEG adsorption onto the glass surfaces, high-resolution XPS measurements were carried out on the model glass surfaces. Figure 3a shows the C1s spectra of silanized glass and PEG-treated model glasses. The peak corresponding to the C-C bond at 285 eV of the silanized glass was one proof of the effective silanization on the surface. The model 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 the C-O bond[18] of PEG. These results clearly indicated that PEG was adsorbed on the glass surface even after rinsing by water without UV irradiation. Furthermore, with increasing hydrophilicity of the solvent (methanol • methanol/water • water), the peak intensity at 286.5 eV increased. Figure 3b shows the quantitative data for the C-O peak area versus the Si_{2p} peak (102.4 eV) after curve-fitting. This data suggests that the adsorption amount of PEG on the silanized glass surface increased with increasing water content in the casting solvent, which agrees well with the contact angle data. Although PEG is amphiphilic, methanol is a better solvent than water. Thus, when the solubility decreases, in other words, when the water content in the solvent increases, PEG prefers to adsorb on the hydrophobic glass surface instead of remaining in solution. Based on all the experimental results obtained from the protein adsorption assay, contact angle measuremants, and XPS analysis, it is concluded that the PEG adsorption tendency onto the glass surface is one of the key factors to determin the characteristics of PEG gel patterned surface prepared by Method B.

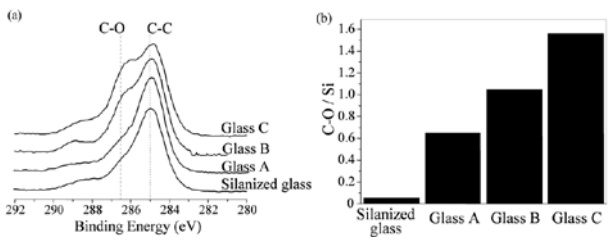

Fig. 3 (a) High-resolution C1s XPS spectra of silanized glass, Glass A, Glass B, and Glass C. (b) C/Si ratio of the glass surfaces, as determined from the XPS spectra. Experimental conditions are described in ESI.

Construction of hepatic spheroid cell microarray on PEG-GEL Pattern

As mentioned in the introduction, a spheroid formation, which is one of the three dimensional cell cultivation techniques, has attracted much attention, because their metabolic functionality retains for a long term[10, 19-21].

In order to evaluate the constructed PEG gel patterned surfaces as a patterning and culture platform for spheroids, the construction of spheroid array with FLC-4 cells was tried on the PEG gel pattern surfaces. Figure 4 shows the pictures of the two inverted FLC-4 adhesion patterns on the constructed PEG gel patterned surfaces. When the PEG gel pattern was prepared by Method A, FLC-4 adhered on the glass area of the constructed pattern surface and formed its cell sheet having 100 μm cavity aligned structure avoiding the PEG gel patterned domains (Figure 4a). On the contrary, when the gel pattern was prepared by Method B, some FLC-4 cells attacked to the PEG gel domain surfaces firstly and formed single-wall cell patterned circles with 100 μm in diameter. Then, floating cells aggregated and formed cell spheroid with uniform size

according to diameter on the constructed pattern surface (Figure 4b). Thus, FLC-4 formed hepatic spheroid on the unmodified PEG gel patterned domains predominately without other feeder cells layer. The cell-incompatibility of the glass area on the constructed PEG gel pattern surface prepared by Method B was maintained for 7 days of culture (Figure S5, see ESI). The amount of albumin secretion of the FLC-4 cells on the constructed spheroids array was extremely higher than that in monolayer culture and almost equal to that from spheroids underlaid with BACEs cultivated on the same PEG gel micropatterned surface (Figure S6, see ESI). These results clearly indicated the usefulness of the constructed spheroid array and PEG gel patterned surface prepared by Method B.

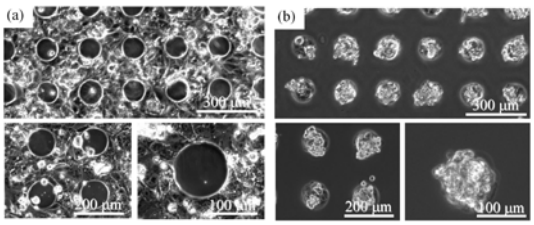

Fig. 4 Phase-contrast micrographs of the FLC-4 sheet with 100 μm aligned cavity structure and cell spheroid array. FLC-4 cultured on a PEG gel pattern surface prepared by (a) Method A and (b) Method B. Experimental conditions are described in ESI.

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 inverted cell adhesion patterns were formed by changing one parameter: the casting solvent used in the film formation step during the preparation of the gel patterns. From the protein adsorption assay, contact angle measurements, and XPS analysis data, it is clear that Methods A and B yielded quite different PEG gel surfaces on the patterned glass surfaces. When methanol was employed as the casting solvent, the PEG gel areas on the constructed 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 co-solvent was used as the casting solvent, the adsorption tendency was totally opposite: the cells adsorbed on the PEG gel area but not 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. Additionally, when FLC-4 were seeded on the PEG gel patterned surface prepared by Method B, cell spheroid formation on the unmodified PEG gel domain circles without feeder cells were observed and they shoewd the highly amount of albumin secretion. This phenomenon is very interesting and make it convenient to construct the cancer cell-based spheroids patterned array, because, in order to construct a cell adhesive PEG surface, further modifications of the PEG gel surface by proteins or peptides are generally needed[22-24]. This technique may widen the scope of cell patterning methodology and the mechanism of the cell attachment on the constructed PEG gel surface prepared by Method B is investigating now.

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Notes and references

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