Materials Science and Engineering C 49 (2015) 256–261

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Arrayed three-dimensional structures designed to induce and maintain a cell pattern by a topographical effect on cell behavior

Takao Saito [a,*], Kay Teraoka [a], Kazuyoshi Ota [b]

[a] National Institute of Advanced Industrial Science and Technology (AIST), 2266-98 Anagahora, Shimoshidami, Moriyama-ku, Nagoya, Aichi 463-8560, Japan
[b] Otake Seisakusyo, Co., 103 Sukanaka, Hottsu-cho, Hashima, Gifu 501-6338, Japan

ARTICLE INFO

Article history:
Received 5 September 2014
Received in revised form 21 November 2014
Accepted 6 January 2015
Available online 8 January 2015

Keywords:
Surface topography
3-D structure
Groove
Cell behavior
Cellular microarray
Scaffold

ABSTRACT

We investigated the ability of the microscale topography of a three-dimensional (3-D) structure arrayed on the surface of a substrate to induce and maintain a cell pattern by controlling cell behavior. Arrayed 3-D structures having different topographical characteristics, i.e., geometry and dimension, were fabricated on the surface of glass substrates by masked sand blasting. Each 3-D structure was designed to have a unit composed of a planar island for cell growth and surrounding grooves exhibiting cell repellency. The principle of the cell repellency is based on the topographical control of cell attachment, spreading, growth, and differentiation by utilizing the spatially restricted microenvironment of the grooves. Grooves with a width of less than approximately 116 μm and a depth of approximately 108 μm formed narrow V-shapes with a dihedral angle of less than approximately 44.4°. Cell culture experiments using osteoblast-like cells demonstrated that these narrow V-shaped grooves had sufficient cell repellency to form and maintain a cell pattern on the surface for at least 14 days. From the present study, arrayed 3-D structures designed to have narrow V-shaped grooves with optimal topographical characteristics for cell repellency are promising for the formation of stable cell patterns for creating novel cell microarray platforms without using conventional protein/cell-repellent chemicals.

1. Introduction

Many biomedical devices such as microarrays, drug delivery systems, biosensors, and scaffolds for tissue engineering have been recently developed for the study and manipulation of biomolecules, and they have proven to be valuable experimental tools for solving many biologically based problems [1]. Microarray technology in particular has become a crucial tool for large-scale and high-throughput biological experiments, as it enables fast, easy, and parallel detection of thousands of addressable elements in a single experiment under identical conditions [2]. Such microarrays of DNA, proteins, and cells are now becoming important tools for exploring genomics, proteomics, and cellomics.

Cellular microarrays have recently received considerable attention because of their important roles in fundamental cell biology, cell-based biosensors, and biological microelectromechanical systems [3]. They are being developed for various cellular analyses of the effects of gene expression, cellular reactions to the biomolecular environment, and cell surface molecule profiles [4,5]. Cellular microarrays offer an additional advantage in their ability to analyze the expression of genes and the function of proteins in a living cell where all of the cellular machinery is present to ensure correct function, thereby facilitating the high-throughput validation of tens of thousands of gene and protein targets [6].

One effective method for creating cellular microarray platforms is based on the patterning of protein/cell-repellant chemicals, such as hydrophilic polyethylene glycol (PEG) derivatives [7], layered on microarray surfaces. These chemicals reduce the non-specific adsorption of cell-adhesive proteins such as collagen, fibronectin, and vitronectin from culture media, and cell patterning is consequently formed on patterned surfaces composed of cell-repellant and cell-adhesive regions. However, the problem with using conventional protein/cell-repellant chemicals is that the formed cell patterns are temporary and break easily within several days because of increased desorption from the surfaces and gradual adsorption of cell-adhesive proteins. Rather than using hydrophilic chemicals such as PEG derivatives, we previously examined block cell adhesion utilizing superhydrophobicity generated by the lotus effect of a perfluoroalkyl isocyanate layer on an oxidized aluminum surface with nanosize topographical features [8,9]. Although a complete cell pattern was formed on the micropatterned perfluoroalkyl isocyanate layer one day after seeding, the formed cell pattern broke after a few days of culture, indicating that the superhydrophobic coating was also not effective for long-term cell repellency.

To investigate cellular responses to physically structured surfaces, the effects of sub-micrometer- and micrometer-scaled surface roughness were previously examined along with the sequential events of cell adhesion, growth, differentiation, and mineralization [10]. The

* Corresponding author.
E-mail address: t-saito@aist.go.jp (T. Saito).

http://dx.doi.org/10.1016/j.msec.2015.01.021
0928-4931/

results suggested that the reduction of cell growth on rough-surfaced substrates was closely related to rounding of cell bodies caused by surface topography at the micrometer scale, accompanied by the disassembly of actin filaments.

Considering the suppression of cell growth on rough-surfaced substrates, in the present study we examined a different strategy to develop cell repellency using tailored 3-D structures rather than chemicals to induce and maintain cell patterns for creating novel cellular microarrays. The 3-D structure consisted of a planar island adequate for cell growth and surrounding grooves for cell repellency induced by surface topography, thus affecting cell shape and spreading through a cell-surface phenomenon called contact guidance [11,12]. The cell repellency of a groove is directly influenced by its geometry and dimension, i.e., its cross-section shape, width, and depth, in addition to surface roughness. Topographical control of cellular responses is a purely physical and biologically non-invasive method without chemicals; therefore, it would be stable against non-specific adsorption of adhesive proteins and effective for long-term cell repellency [13,14]. In the present study, to develop novel cell microarray platforms that maintain cell patterns for a longer duration, we focused on the topographical design of a 3-D structure and examined the effects of topographical characteristics of the grooves, such as geometry and dimension, on cell attachment, growth, differentiation, and pattern formation.

2. Materials and methods

2.1. Substrate preparation

To prepare substrates with arrayed 3-D structures, masked sand blasting was employed to etch the surface of slide glasses (#S1112; Matsunami Glass; Kishiwada, Japan) by using a sand blaster (ELP-4TR; Elfo-tec; Nagoya; Japan). Four types of mask patterns were prepared on photosensitive dry films to generate different 3-D structures. Each mask pattern (A-D) was composed of a square-shaped planar island ($250 \times 250\ \mu m^2$) and surrounding grooves with a width of 50, 100, 150, and 300 μm, respectively. The grooves were etched to have the same depth of 100 μm by sand blasting with a #1200 mesh abrasive with a particle size of approximately 9.5 ± 0.8 μm. Before the cell culture experiments, the surfaces of the etched substrates were thoroughly cleaned by sonication in acetone and then sterilized in 70% ethanol for 10 min.

2.2. Morphological characterization of 3-D structures

Bird's-eye view images of the arrayed 3-D structures fabricated on the surfaces of the slide glasses were taken at a dip of approximately 45°, using a laser microscope (VK-X200; Keyence; Tokyo, Japan). The cross-sectional shapes of the grooves were determined by X-ray micro-computed tomography (μCT; SMX-160LT; Shimadzu; Kyoto; Japan), using a LaB_6 filament and a minimum focus dimension of 0.4 μm. The dimensions of each groove including width, depth, dihedral angle, and radius of curvature at the bottom were measured using images obtained by laser microscopy and μCT.

2.3. Cell culture

The murine osteoblast-like cell line MC3T3-E1 was purchased from Riken Cell Bank (Tsukuba, Japan). The cells were maintained and grown in Minimum Essential Medium-α (α-MEM, Life Technologies, CA) containing 10% fetal bovine serum and antibiotics in a humidified 5% CO_2 balanced-air incubator at 37 °C [10]. The differentiation medium was prepared by adding 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate to the growth medium. Cells with an average diameter of 19.6 ± 1.4 μm and average circularity of 0.84 ± 0.06 were plated onto the sterilized substrates at approximately $3–5 \times 10^4$ cells/cm^2 in 12-well plates and then cultured in growth medium for 1 week and in differentiation medium for the next week. Cell culture experiments of the same conditions were duplicated to investigate cellular responses to the arrayed 3-D structures.

2.4. Observation of cell behavior

2.4.1. Fluorescence microscopy

The cells cultured on the substrates were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized using 0.1% Triton X-100 in PBS for 5 min at room temperature. Actin filaments were stained with 5 U/mL rhodamine phalloidin in PBS containing 1% bovine serum albumin (BSA) for 20 min. The nuclei were stained with 0.3 μM 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI), a DNA binding dye, in PBS for 5 min at room temperature. Fluorescence images of actin filaments and nuclei were obtained using a fluorescence microscope (FM; BX51; Olympus; Tokyo; Japan) equipped with a WUS filter set (excitation filter: 330–385 nm; emission filter: 420 nm) and a WG filter set (excitation filter: 510–550 nm; emission filter: 590 nm), respectively. If necessary, images were electronically combined to generate a co-localized image. Two or more FM images were taken with a different view for each specimen.

2.4.2. Field emission scanning electron microscopy

The cells cultured on the substrates were fixed in 2.5% glutaraldehyde for 2 h at 4 °C, immobilized in 1.0% osmium for 2 h at 4 °C, dehydrated through a series of increasing concentrations (50%, 70%, 80%, 90%, and 100%) of ethanol in water for 30 min, and finally dried with *tert*-butyl alcohol in a freeze dryer. The dried samples were coated with sputtered Pt–Pd. Two or more images with a different view were taken via field emission scanning electron microscopy (FESEM; S-4300; Hitachi High-Technologies; Tokyo; Japan).

2.4.3. Bright field microscopy

Differentiated cells with alkaline phosphatase (ALP) activity were histochemically detected using an ALP kit (86R; Sigma-Aldrich; St. Louis; MO). In brief, the cells on the substrates were fixed with citrate–acetone–formaldehyde solution for 30 s and rinsed with Milli-Q water. Then, the cells were incubated in an alkaline dye mixture for 15 min, rinsed with Milli-Q water, and counterstained with hematoxylin solution for 2 min. After thorough rinsing with Milli-Q water and drying, ALP-positive cells were observed via bright-field microscopy (BX51) and two or more images were taken with a different view.

3. Results and discussion

3.1. Fabrication and characterization of the 3-D structures

From previous results on the effects of surface roughness on the cell adhesion, growth, and differentiation of MC3T3-E1 cells [10], we assumed that cell growth and function could be controlled by structured surfaces at the micrometer scale. Based on this assumption regarding cell-surface interactions, in the present study we focused on how the geometry and dimensions of 3-D structured surfaces affect cell behavior. We designed and fabricated arrayed 3-D structures composed of a planar island and surrounding grooves at the micrometer scale on the surface of a glass substrate, and then examined the effect of the geometry and dimensions of the grooves on cell behavior, i.e., cell attachment, spreading, growth, differentiation, and pattern formation.

Four types of mask patterns were prepared and slide glasses were sand-blasted through these masks. A #1200 mesh abrasive was used to etch different arrayed 3-D structures on the glass surfaces at a depth of approximately 100 μm. Bird's-eye views of the arrayed 3-D structures on slide glasses fabricated by masked sand blasting were obtained via laser microscopy [Fig. 1(A)–(D)]. The surface roughness of the sand-blasted surface was measured to be 0.50 ± 0.05 μm. Cross-section images of the grooves obtained via μCT are shown in the insets of