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Fabrication of micropillar substrates using replicas of alpha-particle irradiated and chemically etched PADC films

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ABSTRACT

We proposed a simple method to fabricate micropillar substrates. Polyallyldiglycol carbonate (PADC) films were irradiated by alpha particles and then chemically etched to form a cast with micron-scale spherical pores. A polydimethylsiloxane (PDMS) replica of this PADC film gave a micropillar substrate with micron-scale spherical pillars. HeLa cells cultured on such a micropillar substrate had significantly larger percentage of cells entering S-phase, attached cell numbers and cell spreading areas.

1. Introduction

Topographical structures affect cell behavior in terms of migration (Frey et al., 2006; Schulte et al., 2009), differentiation (Steinberg et al., 2007), cell deformation (Fu et al., 2010; Davidson et al., 2010), adhesion, morphology, surface antigen display, gene expression (Flemming et al., 1999; Curtis, 2004; Ng et al., 2008a, 2010, in press), cytoskeletal organization and proliferation (Ng et al., 2008b; Baker et al., 2011; Su, 2011).

Recent attempts to introduce optimal topographies for tissue guidance have focused on complex topographies, including discontinuous edged surfaces and pillars (Hamilton and Brunette, 2005; Hamilton et al., 2006b), and cylindrical pillars (Turner et al., 2000). For example, in their studies on nano-scale pillars, Sjöström et al. (2009) showed that small changes in the pillar dimensions (with heights of ~15, 55, or 100 μm) significantly altered hMSC cell behavior, including adhesion, spreading, cytoskeletal formation and differentiation. In another study, Choi et al. (2007) investigated the interactions between human foreskin fibroblasts and silicon nanoposts and nanograte with varying dimensions and patterns. They found that human foreskin fibroblasts exhibited significantly smaller cell size and lower proliferation on needle-like nanoposts, and enhanced elongation with alignment on blade-like nanograte. In the micron-scale regime, many micro-fabrication techniques were deployed to create specified surface topographies to investigate the effects of topography on cellular behavior (Brunette, 1986a,b; Chehroudi et al., 1988; Clark et al., 1990; Hamilton et al., 2006a,b). In particular, micropillar arrays were extensively studied as a potential tool for fabrication of sensors, actuators and tunable wetting surfaces for a whole range of applications including proteomics, genomics and metabolomics (Khandurina and Guttman, 2002; Fang et al., 2008; Panaro et al., 2005). For example, increasing the heights of silicon micropillars from 1 to 10 μm in a square array could affect the mode of fibroblast attachment and could guide the morphology of fibroblasts through increased laminin expression (Su et al., 2010).

Many investigations have revealed distinct effects of micropillar topography on cellular reactions. For instance, creation of micropillars on 2D substrates mimicked 3D microenvironments (Milner and Siedlecki, 2007; Kolind et al., 2010); polydimethylsiloxane (PDMS) pillars with spacing between 5 and 10 μm governed fibroblast migration through actin cytoskeleton rearrangement (Ghibaudo et al., 2009); micropillar geometry affected the differentiation of keratinocytes and mesenchymal stem cells (Steinberg et al., 2007; Mussig et al., 2008; Fu et al., 2010) and malignant transformation of osteosarcoma cancer cells (Davidson et al., 2010).

However, pillar-associated cellular responses might not be always consistent among studies, which might be in part due to the use of different cell types, culture environments and materials. For example, cellular proliferation was not observed with some spatial arrangements of pillars but was observed with others (Ghibaudo et al., 2009; Su et al., 2010). Therefore, more investigations on the topographic effect on cells are needed to provide a clearer picture.

In the present work, we employed a relatively simple and convenient method to fabricate micropillar substrates. Specially

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treated polyallyldiglycol carbonate (PADC) films were used as the cast. PADC films are commonly used solid-state nuclear track detectors (Nikezic and Yu, 2004). Here, a PADC film was first irradiated by alpha particles from a radioactive source through a collimator (to provide more or less normal incidence) for a predetermined period of time (according to the surface density of the pillars required). Chemical etching was then employed to form pores on the irradiated PADC film, and the etching period determined the size and the profile of the pores. PDMS substrates with replicas could be formed from these pores. These micropillar substrates were employed to study the behavior of the cultured HeLa cells, including studies on the S-phase marker expression, attached cell numbers and cell spreading areas, with reference to the behavior of the HeLa cells cultured on the control flat PDMS substrates.

2. Methodology

2.1. Fabrication of micropillar substrates

In the present work, PADC films with a thickness of 1000 μm (Page Mouldings (Pershore) Ltd., Worcestershire) were employed. PADC films were prepared with a size of 2×2 cm^2. The PADC films were divided into two groups for different treatments. The first group of films was irradiated with 5 MeV alpha particles for 30 s using an ^{241}Am alpha-particle source and was then subsequently chemically etched by 6.25 N aqueous NaOH at 70 °C for 30 h, which is the most commonly employed conditions, giving a bulk etch rate of ~1.2 μm/h (Ho et al., 2003). This would lead to creation of micron-scale spherical alpha-particle tracks on the films. The second group of films was unirradiated but etched in exactly the same way as the first group of films, and were employed as "control" films. The etched PADC films were glued inside Petri dishes as casts for the substrates.

The 2×2 cm^2 substrates were cast using PDMS (Dow Corning Corporation, Midland, MI). The spherical pores on the PADC film translated into spherical pillars protruding from the surface of the PDMS substrate. These substrates were employed for subsequent cell culture. The dimensions of the pores in the PADC films formed by etching in NaOH/H_2O, and thus those of the micropillars, could be conveniently calculated using the computer program called TRACK_TEST (Nikezic and Yu, 2006) (also freely available on the webpage: http://www.cityu.edu.hk/ap/nru/test.htm).

2.2. Cell culture

HeLa cervix cancer cells were obtained from the American Type Culture Collection. HeLa cells were maintained as exponentially growing monolayer at low-passage numbers in Dulbecco's modified eagle medium (D-MEM) supplemented with 10% fetal bovine serum, 1% (v/v) penicillin/streptomycin (Gibco, Germany). The cells were cultured at 37 °C in humidified atmosphere containing 5% CO_2. Sub-cultivation was performed every 3 to 4 d. The cells were trypsinized with 0.5/0.2% (v/v) trypsin/EDTA (ethylenediamine-tetra-acetic acid; Gibco), and plated out on the 2×2 cm^2 PDMS substrates with different treatments (as described in Section 2.1) placed inside a 60 mm diameter Petri dish (adjusted to 8×10^5 cells for each dish). Before cell culture, the PDMS substrates were sterilized by submerging in 75% (v/v) ethyl alcohol for 1 h. After washing with PBS, they were immersed in a fresh medium before cell culture. All cells were allowed to plate out on the PDMS substrates for 3 d.

2.3. EdU (5-ethynyl-2′-deoxyuridine) assay

Determination of the S-phase index was carried out using Click-iT^{TM} EdU Imaging Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Briefly, after 3 d culture, the cells were incubated with 10 μM EdU solution at 37 °C for 1 h. The cells were then fixed in 2% paraformaldehyde for 20 min and permeabilization was achieved using 0.5% Triton X-100/ PBS for 20 min. Subsequently, the fixed cells were incubated with the reaction cocktail containing Alexa Fluor® 488- azide for 30 min and then with 1 μg/ml Hoechst 33342 (for nucleus staining) in PBS for another 5 min. Fluorescent images (magnification: 10x) of cells were captured by a fluorescent microscope (Nikon ECLIPES 80i). The Image-Pro Plus software was used for manually counting the total number of cells (Hoechst 33342) and S-phase cells (Alexa Flour® 488) to determine the percentage of cells in the S-phase in the whole cell population.

2.4. Cell number and cell area

In order to count the cell number attached on the PDMS substrates after 3 d cell culture, the attached cells were released by digestion with trypsin–ethylenediaminetetraacetic acid (Invitrogen) and counted using a hemocytometer (Marienfeld, Germany). The cell areas were measured using the ImageJ software (Image processing and Analysis in Java version 1.29x, available on http://rsb.info.nih.gov.ij/).

3. Results

3.1. Micropillars and cultured HeLa cells

The pillar density on PDMS substrates (equivalently the pore density on the PADC casts) was determined as 213 ± 37 mm^{-2}. Using the program TRACK_TEST (Nikezic and Yu, 2006), the dimensions of the etched track were determined as: opening diameter $2r=43.9$ μm and depth $d=15.4$ μm. The corresponding pillars on the PDMS substrate would thus have a base diameter of 43.9 μm and a height of 15.4 μm. Fig. 1 shows the simulated side view of the micropillar on a PDMS substrate. Fig. 2 displayed combined images of expression of EdU marker, expression of Hoechst 33342 and the transmission bright field of HeLa cells cultured for 3 d on (a) a control PDMS substrate, and (b) a micropillar PDMS substrate. The base diamater of the micropillars shown in Fig. 2(b) agreed with the predicted value of 43.9 μm.

3.2. EdU assay, cell number and cell area measurements

A total of 4982 cells (2693 and 2289 cells cultured on control PDMS substrates and micropillar PDMS substrates, respectively) were analyzed for the percentages of cells positively marked by

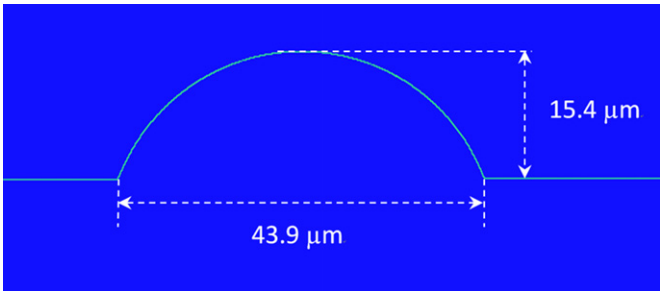

Fig. 1. Simulated side view of the micropillar on a PDMS substrate.

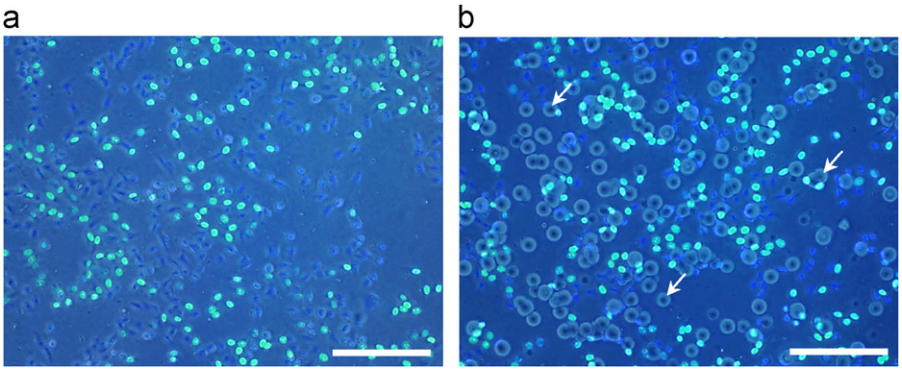

Fig. 2. Combined images of expression of EdU marker (green), expression of Hoechst 33342 (blue) and the transmission bright field of HeLa cells cultured for 3 d on (a) a control PDMS substrate, and (b) a micropillar PDMS substrate. The arrows point to examples of micropillars on the PDMS substrate. Bar: 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

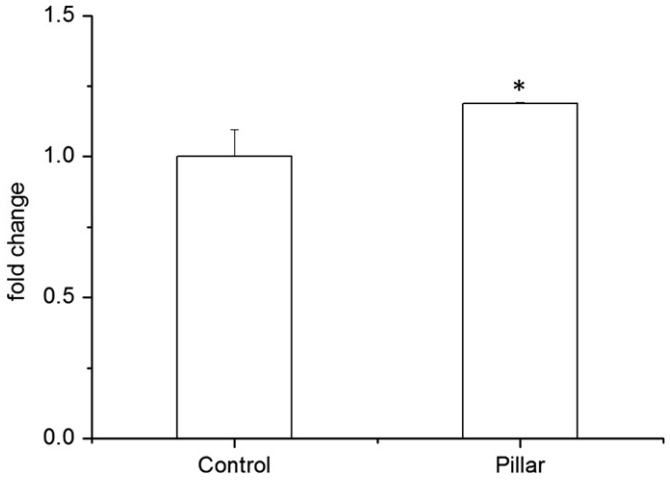

Fig. 3. Fold change in the percentage of cells in the S-phase for cells cultured on micropillar PDMS substrates (with respect to the cells cultured on control PDMS substrates). (The asterisk represents $p < 0.05$, $n = 3$).

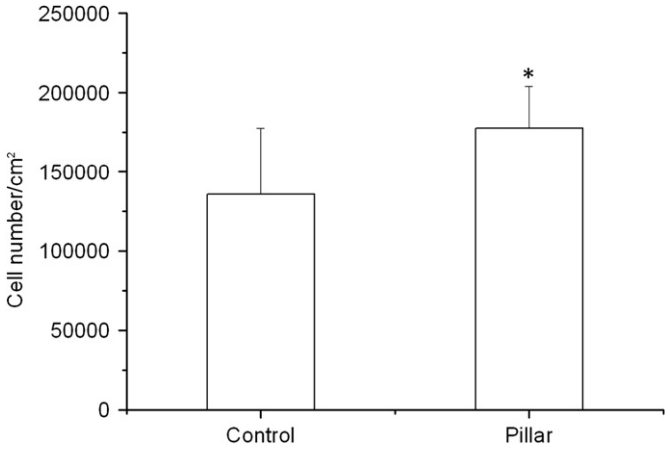

Fig. 4. Number of attached cells (per cm^2) on control PDMS substrates and micropillar PDMS substrates after 3 d cell culture. (The asterisk represents $p < 0.05$, $n = 6$).

EdU assay. Fig. 3 shows the fold change in the percentage of S-phase cells cultured on micropillar PDMS substrates, with respect to the cells cultured on control PDMS substrates. The percentage of cells in the S-phase was significantly higher ($p < 0.05$, $n = 3$) for cells cultured on micropillar PDMS substrates. Fig. 4 illustrates the number of attached cells (per cm^2) on control

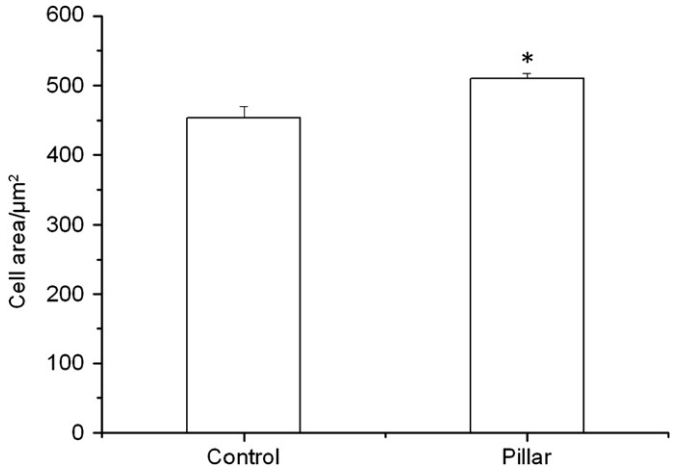

Fig. 5. Average cell areas for cells cultured on control PDMS substrates and micropillar PDMS substrates after 3 d cell culture. (The asterisk represents $p < 0.01$, $n = 3$).

PDMS substrates and micropillar PDMS substrates after 3 d cell culture. The number of cells attached on micropillar PDMS substrates was significantly higher ($p < 0.05$, $n = 6$). A total number of 209 cells (105 and 104 cells cultured on control PDMS substrates and micropillar PDMS substrates, respectively) were analyzed for the cell spreading areas. Fig. 5 displays the average cell areas for cells cultured on control PDMS substrates and micropillar PDMS substrates after 3 d cell culture. The cells cultured on micropillar PDMS substrates had significantly larger areas ($p < 0.01$, $n = 3$).

4. Discussion

In the present work, a novel method for fabricating PDMS substrates with micron-sized pillars was proposed. These substrates were fabricated as replicas of PADC films irradiated with 5 MeV alpha particles and subsequent etching in $NaOH/H_2O$ solution. These micropillar PDMS substrates were employed to study the effect of topography on HeLa cell behavior through EdU assay, determination of attached cell numbers and measurement of average cell areas.

EdU (5-ethynyl-2′-deoxyuridine) is a nucleoside analog to thymidine and is incorporated into DNA during active DNA synthesis (Invitrogen manual MP10338), and it is regarded as an S-phase index. Our results revealed a significant increase in the percentage of cells cultured on micropillar substrates entering the

S-phase when compared to the cells cultured on flat substrates (Fig. 3). This implied more active proliferation of cells on micropillar substrates and explained our results of a significantly larger number of attached cells on micropillar substrates after 3 d culture (Fig. 4). These results were consistent with the findings by Baker et al. (2011) and Su (2011). Baker et al. (2011) also demonstrated substantially enhanced proliferation of fibroblasts on substrates with cylindrical pillars (height ~14 μm and diameter ~10 μm). The pillars fabricated in the present study had a similar height (15.4 μm) but with a different base diameter (43.9 μm) and shape (spherical). Similarly, Su (2011) found an increase in the accumulative cell number of hematopoietic stem cells on substrates with pillars (height=85 μm and diameter=50 μm) in 7, 14, 21 and 28 d after culture. On the other hand, our results showed a significant increase in the spreading areas of cells cultured on micropillar substrates when compared to the cells cultured on flat substrates (Fig. 5). Sjöström et al. (2009) also observed significant increase in the spreading areas of cells cultured on a nano-scale pillar structure (height~15 nm and diameter~28 nm) compared to control surfaces. In conclusion, the micropillars on PDMS substrates were capable of changing the response of cultured cells in terms of cell proliferation and cell spreading area.

The present work offered a new application of PADC films, as casts to fabricate substrates with different pillar structures. Here, we have just fabricated spherical shape pillars as an example. In fact, this method provides a whole range of flexibilities in generating substrates with pillars having different characteristics such as diameters, heights and shape, which can be achieved by varying the etching conditions, or the energy of the alpha particles or both in creating the PADC casts. Apart from micron-scale pillars, nano-scale pillars can also be fabricated. Different pillar profiles can be conveniently designed with the help of the TRACK_TEST computer program (Nikezic and Yu, 2006). Combinations of pillars with different profiles are also possible and can be conveniently introduced to the substrate surface. The pillar density can be simply controlled by the alpha-particle irradiation time in creating the PADC casts. Substrates with different types of pillars will be extensively studied in the future.

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