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Convenient fabrication of three-dimensional cell-culture substrates through introduction of micrometer-size pores on polyallyldiglycol carbonate polymer films

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ABSTRACT

We explored the fabrication of three-dimensional (3D) substrates by creating micrometer-size pores on polyallyldiglycol carbonate (or PADC) polymer films through irradiation of the film by alpha particles and subsequent chemical etching. HeLa cells cultured on these 3D substrates were observed using scanning electron microscope. Multiple directions and multiple layers of HeLa cells were observed to have grown in the pores, with normal nuclei and cell membranes as well as good cell spreading. For the cells cultured in 3D substrates with or without additional small pores, no significant differences were observed between their vinculin expression profiles, which were in contrast to the observation made for cells cultured on 2D substrates showing that small pores could enhance vinculin expression. The presence of the large pores and/or the enhanced biocompatibility of the substrate in the present experiments might be the reasons. The protrusions of cells were confined by the small pores, which was similar to the observation made for cells cultured on 2D substrates.

1. Introduction

In vitro experiments rely heavily on tissue culture, e.g., to study the cell differentiation, proliferation, function, etc. These *in vitro* experiments usually involve flat culture substrates, e.g., through the use of Petri dishes and flasks, and are thus convenient for routine growth of cells. However, such conventional cell culture will normally generate only two-dimensional (2D) cell monolayers. Such 2D monolayers will lead to highly abnormal geometric and mechanical pressures on many types of cells, which are far from the realistic conditions and complexities of three-dimensional (3D) tissues [1].

It is well established that cells in tissues connect to each other and to the extra-cellular matrix (ECM). Receptor complexes on the surface of cells facilitate interactions with their neighbors, with the ECM and other exogenous factors, to enable cells to interpret the multitude of physical and biochemical cues from the immediate environment. Moreover, 3D mammalian cell culture promotes normal epithelial polarity and differentiation [1]. Considering this intricate mechanical and biochemical interplay, important biological properties may be missed if the cells are studied within a 2D culture system. The lack of dorsal anchorage points in 2D cultures affects the balance between cell spreading or retracting, creating an unnatural stimulatory environment for the organization of lamellipodia, stress fibers and focal adhesions. This imbalance leads the cells to spread out in an extreme manner. It has been found that the context in which a cell is grown matters and changing the environment can radically alter the behavior of cells [2]. In relation, there have been many studies on the advantages of 3D cell culture environment when compared to the 2D case. Cells have been shown to move and divide more quickly, and to have a characteristically asymmetric shape as those in living tissues [3]. The spatial arrangement of ECM receptors in 2D is mainly concentrated on the ventral surface, whereas in 3D cultures they are spread over the entire surface.

Evidences have suggested that modification of cell growth conditions can radically affect the behavior of cells in response to chemical reagents [4]. In addition, researches have shown that cancerous breast cells growing in 3D have patterns of gene expression and other biological activities that more closely reflect the activities of cells in living tissues [5,6]. There is also a substantial amount of evidence that cells growing in 3D are more resistant to cytotoxic agents than cells grown in monolayer or in a dispersed culture [7]. Studies have demonstrated an elevated level of drug resistance of spheroids culture compared with cells in monolayer [7]. Initially, investigators attributed the drug resistance of spheroids to poor diffusion of the drugs to interior cells but now it has been proved that the 3D culture itself accounts for

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drug resistance [8,9]. Furthermore, culturing human pluripotent stem cells on 3D scaffolds can markedly affect the cell behavior [10,11] by offering growing tissues the opportunity to exploit additional space and form alternative interactions with neighboring cells, noting that interactions are restricted in cultures grown as 2D monolayer.

As such, *in vitro* experiments involving 2D cell monolayer will inevitably suffer from the limitation that they do not adequately represent the functions of 3D tissues, and the relevance of results from *in vitro* experiments to *in vivo* animal or human studies is thus continually challenged. Accordingly, it has always been desirable to design and fabricate convenient and economical substrates which can allow the cells to grow in a 3D manner, so as to mimic functions and responses of cells in real tissues. For examples, 3D cell cultures have been used extensively recently, particularly in tissue engineering applications, e.g., cell-seeded scaffolds [12] and patterned cocultures [13] as well as in directing cell fate and differentiation [14], to mimic native micro-environments. This is a crucial step for improving the predictive accuracy or in understanding cell metabolism and other cell behaviors [15,16].

In the present work, we have explored the fabrication of such a 3D substrate by creating micrometer-size pores on polyallyldiglycol carbonate (or PADC) polymer films through irradiation of the film by alpha particles and subsequent chemical etching. PADC film is one commonly used solid-state nuclear track detector (SSNTD). There was a recent review on SSNTDs and their applications [17]. PADC is sometimes commercially available under the name CR-39, which is widely used in different branches of sciences. In particular, as they can record alpha particles, thin PADC films with a thickness of 10–20 μm have been proposed as cell-culture substrates for alpha-particle radiobiological experiments (e.g., [18–20]). Previous experiments have been performed to study the topographic effect on cell behavior (e.g., [21]). In particular, it has been established that changes in the surface topography of SSNTDs through chemical etching or superficial pore formation can enhance the biocompatibility [22,23].

2. Methodology

2.1. Characterization of PADC films

In the present work, "thick" PADC films with a thickness of 1000 μm from Page Mouldings (Pershore) Limited, Worcestershire were employed. Although in reality we will use "thin" PADC films with a thickness of 10–20 μm, the physical and chemical properties of thick and thin PADC films are the same, with the thick films being more convenient to handle.

PADC films with a size of $2 \times 2\,cm^2$ have been prepared as substrates for cell cultivation. In the present experiments, there were two types of treated PADC films. Both types of treated films were irradiated with 5 MeV alpha particles using an ^{241}Am alpha-particle source and were 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 [24].

One type of treated films was etched for 30 h. The other type of treated films were first etched for 27 h, then irradiated by 3 MeV alpha particles for 6 h (with an average track density of about 830,000 tracks cm^{-2}), and subsequently further etched for 3 h, so the aggregate etching time was also 30 h. As a final step, both types of treated (and etched) PADC films were further etched for 5 min in 1 N NaOH/ethanol at 40 °C (with a bulk etch rate of ~9.5 μm/h [25]). The PADC films were etched in NaOH/ethanol in the end because Li et al. [18] found that this step would bring about better biocompatibility.

Upon the chemical etching steps, the 5 MeV alpha-particle irradiation with 30 h etching will lead to larger pores while the 3 MeV alpha-particle irradiation with 3 h etching will lead to smaller pores. The dimensions of large pores and small pores were measured experimentally. Those for the large pores were determined using a surface profilometer (Form Talysurf PGI Profilometer, Taylor Hobson, England), which was a contact stylus instrument based on a phase grating interferometric (PGI) transducer. During measurements, a computer-controlled stylus scanned slowly across a surface of the specimen. The movement of the stylus was converted into an electrical signal by the computer and the profile of the scanned surface was generated. The small pores were tracks in the sharp phase, which was described in a previous paper [23]. For a comparison, the opening diameters of pores in PADC films formed by etching in $NaOH/H_2O$ alone were calculated using the computer program called TRACK_TEST [17] (also freely available on the webpage: http://www.cityu.edu.hk/ap/nru/test.htm). It is remarked here that the opening diameters can no longer be simulated using TRACK_TEST after a further etching in NaOH/ethanol for 5 min, since the *V* function (ratio between the track etch rate and the bulk etch rate) of PADC in NaOH/ethanol, which is required by the TRACK_TEST program for computation, is unknown. On the other hand, the dimensions for the small pores were determined from their lateral images after polishing the edge of the films as described in a previous paper [23].

2.2. Cell culture

HeLa cervix cancer cells were obtained from the American Type Culture Collection. HeLa cells were cultured on the $2 \times 2\,cm^2$ PADC films with different treatments as described in Section 2.1. Before cell culture, the PADC films were sterilized by submerging in 75% (v/v) ethyl alcohol for 2 h and then immersed in fresh medium for 24 h before cell culture. These films were then used for HeLa cell culture. 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 3–4 d. The cells were trypsinized with 0.5%/0.2% (v/v) trypsin/EDTA (ethylenediamine-tetra-acetic acid; Gibco), adjusted to 4.5×10^5 cells in 3 ml medium and plated out on the PADC films placed inside a 35 mm diameter Petri dish. All cells were allowed to plate out on the PADC films for 1 d.

2.3. Scanning electron microscope observation

Scanning electron microscope (SEM) was used in order to have a better view of cells seeded on large pores. Substrates with large pores were immersed in 0.1 mg/ml polylysine in MilliQ before cell seeding to improve cell adherence. The cells cultured on PADC films with different treatments were fixed in fixation buffer (2% paraformaldehyde, 2.5% Gluta, 0.1 M cacodylate buffer, pH 7.2, 0.05% $CaCl_2$) overnight at RT for 2 h. The fixed cells were washed with washing buffer (0.1 M cacodylate buffer (pH 7.2)) for 5–10 min five times at RT. Then, the cells were post-fixed with 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.2) at RT for 2 h. The post-fixed cells were transferred from the washing buffer into the water and then dehydrated gradually with ethanol. After dehydration, cells were gradually transferred to acetone at RT and then critical-point-dried in liquid CO_2. The dried PADC films were mounted, with cells at the surface top, on aluminum stubs with double side carbon tape (Nisshin EM Co. Ltd., Tokyo) and then gold-coated by using a sputter coater. The samples were then