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Short communication

Cell growth on 3D microstructured surfaces

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ABSTRACT

Chinese Hamster Ovary (CHO) cell cultures were grown on surfaces lithographed with periodic 3D hexagonal microcavity array morphology. The range of microcavity size (inscribed circle diameter) was from 12 μm to 560 μm. CHO cells were grown also on flat surfaces. The characterization was performed with respect to cell growth density (number of nuclei per unit area) by fluorescence optical microscopy and evaluated by correlation function analysis. We found that optimum microcavity radius was 80 μm, concerning to the maximum cell growth density, being even greater than the growth density on a flat (unstructured) substrate of the same material. This finding can be important for optimization of biotechnological processes and devices.

1. Introduction

The chemical and structural similarities of cells lead us to search for systematics in their architecture and components [1]. It is known that cell growth is usually anchorage dependent and requires attachment to a solid surface [2–4], and cell adhesion to biomaterials with a suitable surface is fundamental to many biotechnological processes [5,6]. Numerous human and veterinary use pharmaceuticals, such as human tissue plasmid activator [7,8] and viral vaccines (hepatitis [9], rabies [10], influenza [11], polio, rotavirus, food engineering [12] and mouth disease [12,13]) are produced by anchorage dependent cells. Geometrical and mechanical properties of the cell microenvironment have great impact on cell morphogenesis and function, and can interfere with cell cytoskeleton architecture, polarity, migration, division, growth and differentiation. Cell behavior can be regulated by interaction between neighboring cells and by interaction with the extracellular matrix (ECM) [14,15]. These environmental conditions are vital for homeostasis maintenance, and their deregulation can lead to loss of integrity or even cell death [16]. Thus cell growth on microstructured surfaces is an important field of investigation.

The literature reports a number of studies of cell cultures grown on surfaces modified by nano/micro-patterning of different ECM receptors in selected areas [17–24]. These studies have explored cell growth on various kinds of patterning formed by a variety of techniques, and have looked at how the cell culture responds to these features. However, cell growth on 3D microstructures that tend to isolate cells within 3D "microcavities" - a kind of microstructuring that differs fundamentally from the prior work - has been not reported previously. The work summarized here makes use of electron beam lithographed hexagonal microstructures as the surface morphology on which the cells are grown.

The objective of this work was investigating cell growth on chemically homogeneous microcavity patterns, monitoring the growth behavior of the cells due to morphology of the surface. The results of this work can contribute for scaffolds design [25–27] and for optimal conditions for cell growth in general sense.

2. Materials and methods

2.1. Surface microfabrication

Chinese Hamster Ovary (CHO) cells were cultured on 3D hexagonally microstructured substrates. Fluorescence optical microscopy (FOM) and correlation function analysis were used to characterize the growth. Periodic microstructures were generated on SU-8 (2005 from Micho Chem, resin used for electron beam lithography – electron resist) surfaces by electron beam lithography. The morphology was composed of hexagonal cavities; see Fig. 1, where R is the radius of a circle inscribed within the hexagonal microcavity, h the cavity depth and β the wall thickness between cavities. The cavity aperture $2R$ was varied for different substrates, with values of 12, 30, 80, 280 and 560 μm, generating samples referred to here as HEX-12, HEX-30, HEX-80, HEX-280 and HEX-560, respectively. The cavity depth h was kept constant at $h =$ 3 μm, and the wall thickness β varied according to the cavity cell size

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2R. The parameters used for electron beam lithography were 15 pA beam current, 0.07 nC/cm exposure dose, and 10 mm working distance.

2.2. CHO cell growth

The cells were stored in liquid nitrogen (freezing solution 10% DMSO) in aliquots of 1×10^6 cells/mL, and thawed and seeded in monolayers into flasks of 25 cm^2 (Corning), in HAM-F10 (Invitrogen) medium supplemented with 10% fetal bovine serum (Cultilab) and antibiotics (streptomycin, 1% and penicillin 1%). Cell cultures were incubated in humid 5% CO_2 atmosphere at 37 °C until reaching approximately 90% confluence, when they were subcultured. At that time, the medium was removed and 5 mL of Hanks 1X solution (0.4 g KCl, 0.06 g KH_2PO_4, 0.04 g Na_2HPO_4, 0.35 g $NaHCO_3$, 1 g glucose, 8 g NaCl, H_2O q.s.p. 1000 mL) were added for 2 min. The Hanks solution was removed and 2 mL of trypsin 0.25%/EDTA 1X (Invitrogen) were added for 5 min, until the cells detached. Subculture was performed splinting 1 confluent culture flasks to 2 sub-confluent culture flasks (1:2 ratio) every 24 h in culture flasks of 25 cm^2. For the studies carried out, experimental design cells were always between the 3rd and 5th passages. Cell viability on the surfaces was evaluated beforehand by the trypan blue (Merck) exclusion test. The number of dead cells (apoptosis/necrosis) was about 0.7%, which is not significant.

2.3. Fluorescence optical microscopy

After 24 h incubation, cells were fixed and their nuclei labeled with DAPI fluorophore as follows. The attached cells were washed three times with PBS 1X 1 M at 37 °C and fixed using 4% paraformaldehyde for 15 min at room temperature (RT). Cells were permeabilized by washing three times with 0.2% Triton X100 (Sigma) in PBS for 5 min at RT. The cells were then washed again three times with PBS and stained with Prolong Gold DAPI (Life Technologies). A volume of 20–30 μL of this suspension was placed onto the substrate surface and covered with a coverslip. Samples were allowed to set in the dark, at room temperature, for 24 h before analysis. Nucleus morphology was observed using an epifluorescence microscope (Olympus BX 51 with U-RFL-T fluorescence module), with light source at 350 nm excitation wavelength and with a CCD camera (Qcolor 5) for image acquisition.

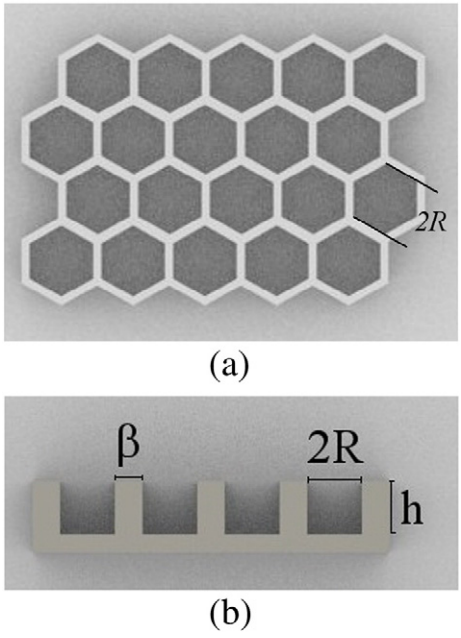

Fig. 1. Schematic of the periodic microstructures used. The morphology was composed of hexagonal cavities, shown (a) in top view, and (b) in cross-section. *R* is the radius of the inscribed circle, *h* the cavity depth, and *β* the wall thickness between cavities.

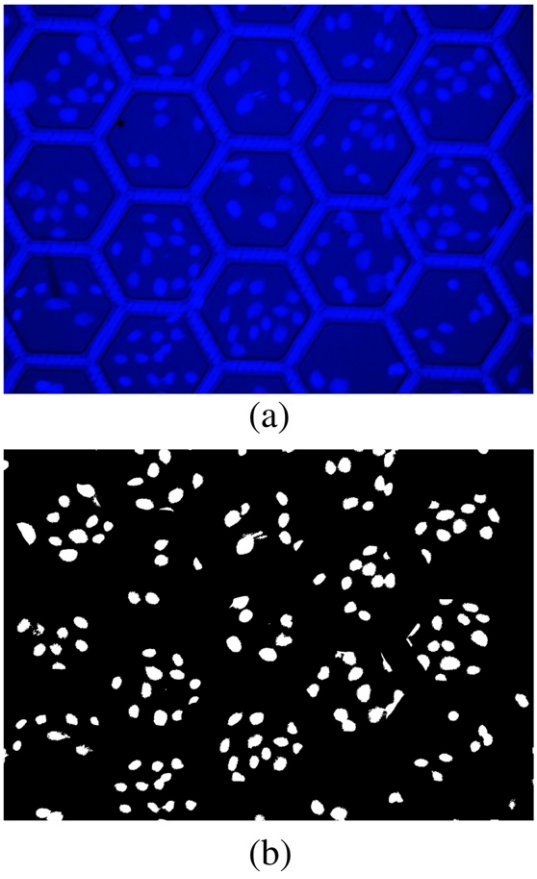

Fig. 2. Fluorescence optical microscopy images. (a) Original captured image and (b) Binarized image. The images have size of 437 × 328 μm^2 at 0.17 μm/pixel.

The nucleus of the cell was considered intact when it was glowing brightly and homogeneously; apoptotic nuclei were identified by their condensed chromatin at the periphery of the nuclear membrane or by totally fragmented nuclear bodies [28]. Cell growth was evaluated by counting the number of nuclei per unit area on each substrate.

2.4. Image processing

The FOM images (Fig. 2(a)) were processed by extracting the blue channel using ImageJ software [29–31] or Fiji software [32], after which the resulting images were in grayscale, allowing correlation analysis. The color of the structures was first set to the color of the background, and binarization then done. The final image was evaluated by correlation function analysis, where the white cells were associated with a "1" value and the black background associated with "0" (Fig. 2(b)).

2.5. Statistical analysis

For each surface pattern size was performed three identical experiments, generating groups of three samples. Normality tests for

Table 1
Parameters of the hexagonal microstructures lithographed on SU-8 as obtained by SEM and AFM measurements.

Sample	Diameter, *2R* (μm)	Wall thickness, *β* (μm)	Height, *h* (μm)
HEX-12	12	1.3	3
HEX-30	30	2.0	3
HEX-80	80	3.5	3
HEX-280	280	6.5	3
HEX-560	560	9.0	3